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# STEROIDS AND THE STIFFNESS SYNDROME IN GUINEA PIGS

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Wulzen and Bahrs (1-3) first demonstrated the "stiffness syndrome" in guinea pigs. Van Wagtendonk and coworkers (4-7) reported studies of the metabolic changes resulting from the deficiency of the fat-soluble factor involved. The isolation of the active factor from raw cream and cane juice has been described by van Wagtendonk and Wulzen (8, 9). However, several investigators have been unable to demonstrate the characteristic deficiency symptoms in guinea pigs (10, 11).

Using a diversity of diets, we were able to produce deficiency symptoms in guinea pigs which were apparently identical with those described by the Oregon State College group. Several synthetic diets, the skim milk diet of van Wagtendonk (4), and a commercial type of pelleted diet, were used for the study of this deficiency disease. The syndrome appeared the most rapidly and severely on the pellet diet. This is partly due to the poor growth and physical condition of the animals fed the synthetic or skim milk diets and partly to some property of the pellet diet which markedly induces a severe deficiency. This phenomenon may be caused by the presence in this diet of the "antagonistic factor" mentioned by van Wagtendonk and Wulzen (9).

## EXPERIMENTAL

The pellet diet was used in all of the experiments reported here. Its composition is given in Table I. The guinea pigs were obtained from various commercial breeders and weighed from 200 to 400 gm. at the start of the experiment. The animals were housed in individual cages on raised screens and were given food and water *ad libitum*.

Signs of muscle stiffness usually developed in 1 to 3 weeks, depending on the age and source of the guinea pigs. In fact some animals were already deficient when we received them, indicating that the previous dietary history of the animals is an important factor in determining the time of onset of the disease in any given experiment.

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The procedure used for determining the severity of stiffness is the same as that described by van Wagtendonk and Wulzen (9). The fore leg of the animal is extended posteriorly along the body wall, supported from below by the operator's fingers, and held rigid by downward pressure on the olecranon process with the operator's thumb. By use of the other hand, the paw is flexed upward by gentle pressure. This bending at the wrist to a 90° angle is accomplished very easily in a normal animal. With deficient animals, bending becomes more difficult and the angle to which the paw can be bent decreases. In very severely deficient animals, the paw will not bend at all.

TABLE I  
*Composition of Basal Diet\**

	<i>lbs.</i>
Ground wheat.....	20
“ whole yellow corn.....	15
Feeding oat meal.....	20
Wheat bran.....	5
Soy bean oil meal (41% expeller).....	20
Linseed oil meal.....	5
Fish meal (60% vacuum).....	2
Alfalfa leaf meal (dehydrated 20%).....	10
Bone meal (steamed).....	1
Limestone (feeding).....	1
NaCl.....	0.25
Delsterol (900,000 i.u. vitamin D).....	0.125
Ascorbic acid†.....	<i>gm.</i> 12

\* Manufactured by the Derwood Mills, Derwood, Maryland.

† Each animal received 20 mg. of ascorbic acid orally three times a week as additional ascorbic acid.

The 1+ to 4+ system, described in detail by van Wagtendonk and Wulzen (9), was used to indicate the severity of the stiffness. A 3+ animal was found most suited for assay purposes. In the 3+ deficiency, the paw bends with some difficulty to approximately a 60° angle. Curative tests were used entirely. Responses to therapy were designated ++, +, ±, or —, depending on whether complete, marked, very little, or no alleviation of the symptoms occurred in the 5 day test period. Only ++ or + responses were regarded as positive and a supplement was considered active when 50 per cent or more of the animals in a group gave this response.

The compounds to be tested were dissolved in refined cottonseed oil and daily doses were given orally for 5 days to test animals showing a 3+ deficiency. Most compounds were tested at a level of 5 γ per day. Final read-

TABLE II  
New Esters of Ergostanol

Ester	M.p. °C.	Empirical formula	Analytical data	
			Calculated	Found
			per cent	per cent
Formate	111 -112.5	$C_{29}H_{50}O_2$	C 80.87, H 11.70	C 80.50, H 11.98
Propionate	150 -151	$C_{31}H_{54}O_2$	" 81.16, " 11.87	" 81.02, " 11.88
Isovalerate	111.5-113	$C_{33}H_{58}O_2$	" 81.42, " 12.01	" 81.71, " 12.14
Isocaproate	97 - 98	$C_{34}H_{60}O_2$	" 81.33, " 12.08	" 81.38, " 12.42
Pelargonate	99 -100.5	$C_{37}H_{66}O_2$	" 81.85, " 12.25	" 81.87, " 12.52
Laurate	102 -103	$C_{39}H_{70}O_2$	" 82.04, " 12.36	" 81.76, " 12.74
Palmitate	105 -106*	$C_{44}H_{80}O_2$	" 82.27, " 12.58	" 82.14, " 12.91
Stearate	103 -105*	$C_{46}H_{84}O_2$	" 82.54, " 12.68	" 82.15, " 13.03
Ethyl carbonate	127 -128	$C_{31}H_{54}O_3$	" 78.42, " 11.47	" 78.49, " 11.41
Ethyl adipate	113.5-115	$C_{38}H_{68}O_4$	" 77.36, " 11.18	" 77.47, 77.56, H 11.76, 11.69
Cinnamate	183.5-184.5	$C_{37}H_{58}O_2$	" 83.40, " 10.59	" 83.41, H 10.94
Cyclohexane carboxylate	146 -146.5	$C_{33}H_{50}O_2$	" 81.97, " 11.79	" 82.21, " 12.16
Phenylurethan	173.5-179.5	$C_{35}H_{55}O_2N$	" 80.56, " 10.62, N 2.63	" 81.06, 80.89, H 10.93, 11.18, N 2.73
$\alpha$ -Tetraacetylglucoside	195 -196.5	$C_{45}H_{78}O_{10}$	" 68.82, " 9.36	" 68.58, H 9.69

\* Cloudy melt.

STEROIDS AND STIFFNESS SYNDROME

TABLE III

*Action of Steroids on Stiffness Syndrome*

Compounds tested	Level fed, per day	No. of times tested	5 day response (No. of guinea pigs)				Activity No. positive Total
			++	+	±	-	
Ergostanol	5 γ	4	5	3	3	8	8/19
"	25 "	1	4	1		1	5/6
Ergostanyl acetate	1 "	3	5	5	3	8	10/21
"	5 "	12	24	16	12	12	40/64
"	5 "	1	3	2			5/5
"	50 "	1	2		1		2/3
"	100 "	1	1	3			4/4
"	5 "	1			1	6	0/7
"	5 "	1	3	1		1	4/5
"	5 "	2	4	6	4	2	10/16
"	5 "	1				8	0/8
"	5 "	1	1	3	1	2	4/7
"	5 "	1		3	1	4	3/8
"	5 "	1	1	3	2		4/6
"	5 "	1			3	4	0/7
"	5 "	1			2	6	0/8
"	5 "	1		2	1	4	2/7
"	5 "	1			1	7	0/8
"	5 "	1	3		1	1	3/5
"	5 "	1		1		6	1/7
"	5 "	1		5	2	1	5/8
"	5 "	1		1	3	4	1/8
"	5 "	1			2	5	0/7
α-Ergosterol	5 "	1	1		2	1	1/4
"	100 "	1	1	2		1	3/4
β-Ergosterol	5 "	1	1		1	1	1/3
"	100 "	1		1	1	1	1/3
γ-Ergosterol	5 "	1		2	1	1	2/4
γ-Ergostenyl acetate	5 "	1			3	1	0/4
Dehydroergostenyl acetate	5 "	1	1		1	2	1/4
"	100 "	1	1	2		1	3/4
γ-Dihydroergosterol	5 "	1		2	2	4	2/8
γ-Dihydroergosteryl acetate	5 "	1				5	0/5
Ergosterol	5 mg.	1		2	1	1	2/4
"	10 "	1			1	3	0/4
"	10 "	1				2	0/2
Dehydroergosteryl acetate	5 γ	1	1		1	2	1/4
"	100 "	1	2	2			4/4
β-Ergosterol oxide	5 "	1		2	2	4	2/8
Epiergostanol	5 "	1				4	0/4
Epiergostenyl acetate	5 "	1			2	2	0/4
Chlorergostane	5 "	1				8	0/8

TABLE III—*Concluded*

Compounds tested	Level fed, per day	No. of times tested	5 day response (No. of guinea pigs)				Activity No. positive Total
			++	+	±	—	
Ergostane	5 $\gamma$	1			4	2	0/6
Cholesterol	5 mg.	1			1	3	0/4
Cholesteryl propionate	5 $\gamma$	1			2	5	0/7
Cholestanol	5 "	1			2	3	0/5
Cholestanyl acetate	5 "	1		1		3	1/4
7-Ketocholestanyl acetate	5 "	1		1	3	3	1/7
7-Ketocholesteryl "	5 "	1			2	6	0/8
Cholestanone	5 "	1				7	0/7
$\Delta^4$ -Cholestenone-3	5 "	1			2	6	0/8
Stigmastanol	5 "	1		2	1	2	2/5
Stigmastanyl acetate	5 "	1			1	4	0/5
Stigmasteryl "	5 "	1			1	7	0/8
Phytosterol	5 mg.	1	1		1	2	1/4
$\alpha_1$ -Sitostanol†	5 $\gamma$	1	1	1		6	2/8
$\alpha_1$ -Sitosterol†	5 "	1	1	1	2	3	2/7
$\beta$ -Sitosterol	5 "	1	1		1	4	1/6
$\alpha_3$ -Sitosterol	5 "	1		1	2	4	1/7
Cholanic acid	5 "	1		1	2	2	1/5
Cholic acid	1 mg.	1			1	4	0/5
Methyl cholate	5 $\gamma$	1			3	5	0/8
Lithocholic acid	5 "	1	1	1	1	3	2/6
Desoxycholic acid	5 "	1		2		5	2/7
3-Ketocholanic acid	5 "	1				7	0/7
Methyl 3( $\alpha$ )-12-ketocholanoate	5 "	1		2	2	2	2/6
Estrone	2 "	1	1	1	1	4	2/7
Crystalline vitamin D <sub>2</sub>	5 "	1			1	5	0/6
Vitamin D <sub>3</sub> (delsterol)	5 "	1	1	1	1	3	2/6
Dihydrotachysterol	5 "	1			2	7	0/9
Crystalline "antistiffness" factor	1 "	5	7	5	2	6	12/20
" " "	2 "	3	4	6	1	7	10/18
" " "	5 "	1	1	1	1	1	2/4
Negative controls		23	1	14	19	79	15/113

\* Injected intraperitoneally.

† Bernstein *et al.* (12) have indicated the possibility that  $\alpha_1$ -sitosterol may be non-steroidal.

ings were taken late on the 5th day, and the responses were read by the operator, who did not know from which group the animals were taken. Cottonseed oil was found to be inactive.

In the course of testing various natural materials and extracts, "anti-stiffness" activity was found in crude sterol preparations made from *Penicillium notatum*. A number of pure steroids were then assayed and certain

ones were found to be very potent. This paper is concerned only with tests made on pure compounds.

Of the pure compounds assayed biologically, fourteen are new esters of ergostanol. The physical properties of these compounds which were prepared by the standard procedures are summarized in Table II.

The biological activities of the compounds tested are presented in Table III. The individual tests are given. The control groups are also listed to indicate variations in the assay over the test period. In addition to the known compounds tested, a sample of crystalline "antistiffness" factor, kindly supplied by Dr. A. L. Caldwell, was also assayed several times and served as a positive control.

### *Results*

The most active compounds tested were found to be ergostanol and certain of its esters. Since ergostanyl acetate appeared to be more active than the free sterol, our attention was mainly directed to this derivative.

On the basis of this preliminary study of a large number of steroids, certain correlations between activity and structure were suggested. Obviously more experiments will be required to substantiate some of the following relationships. (1) Sterols having the ergostane carbon skeleton were most active, since, *e.g.*, cholestanol, stigmastanol, bile acids, and estrone were inactive. (2) Replacement of the hydroxyl group at the C-3 position of ergostanol with chlorine or hydrogen gave inactive compounds. (3) Inversion of the hydroxyl group of ergostanol decreased the activity, since epiergostanol and its acetate were inactive. (4) Complete saturation of the ergostane ring system was found to be necessary for maximum activity since ergosterol was inactive. It would appear in this connection that compounds of less degree of saturation in the ergostane series are less active than ergostanol and its acetate. (5) The crystalline "antistiffness" factor showed good activity in our tests. While a precise comparison of activity is difficult at this time, its activity was approximately the same as that of ergostanyl acetate.

### DISCUSSION

It cannot be said that any one of the pure compounds which we have found active is identical with the crystalline "antistiffness" factor isolated by van Wagtenonk and Wulzen, since we have not isolated any material from a natural source and the chemical nature of their active substance has not been revealed. However, under our experimental conditions, ergostanyl acetate is approximately as active as their crystalline material.

The degree of structural specificity exhibited by the series of compounds tested is of considerable interest and has been discussed above.

## SUMMARY

1. A severe deficiency of the "antistiffness" factor was produced in guinea pigs fed a natural diet.

2. Of the fifty-nine steroids tested, certain esters of ergostanol showed the greatest activity. The relationship between structure and activity has been discussed.

3. The preparation and physical constants of fourteen new esters of ergostanol have been presented.

4. A sample of the crystalline "antistiffness" factor isolated by van Wagtendonk and Wulzen was assayed and found to be highly active.

We are indebted to Dr. W. J. van Wagtendonk and Dr. R. Wulzen, Oregon State College, for very helpful advice. We are also indebted to Dr. A. L. Caldwell of Eli Lilly and Company for a sample of the crystalline "antistiffness" factor, and to Professor Everett S. Wallis, Princeton University, for specimens of  $\alpha_1$ -,  $\alpha_3$ -, and  $\beta$ -sitosterol and  $\alpha_1$ -sitostanol.

We wish to thank Messrs. Louis Brancone, William Fulmor, and Samuel Modes for the microanalyses, and Mr. Karl J. Sax for assistance in the preparation of several of the compounds.

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# THE INFLUENCE OF DIETARY PROTEIN, METHIONINE, AND CYSTINE ON ACCELERATED VITAMIN C EXCRETION IN THE RAT

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The rat does not require an exogenous supply of vitamin C for growth and does not develop scurvy when kept on a synthetic diet for many generations. The ability of the rat to synthesize vitamin C was proved by the demonstration that scorbutic guinea pigs could be cured by the feeding of liver from rats maintained for a long period of time on a diet free from this vitamin (1). It was subsequently shown (2-4) that the urinary excretion of vitamin C by rats could be greatly increased by certain organic compounds and that this excretion represented an accelerated synthesis of the vitamin. The chemical determinations of urinary vitamin C were in close agreement with guinea pig assays performed on the same samples.

The mechanism by which these agents exert their action is still obscure, although because of their widely differing chemical structures it is certain that the stimulating substances themselves do not serve as precursors of vitamin C. Thiamine has been found to be necessary for maximal response to these agents (5). Sodium pyruvate intraperitoneally administered has been shown to enhance the response of rats to chloretone (6).

The present experiments were undertaken as the first step in the study of the mechanism of stimulated vitamin C excretion. The effects of altering the dietary level of protein on the responses to sodium phenobarbital and chloretone, two of the most potent stimulating agents, were studied, and specific effects of methionine and cystine were observed under the dietary conditions employed. Preliminary reports of this work have been made (7, 8).

## EXPERIMENTAL

The rats employed in these studies were of the Wistar strain and were obtained from a stock colony maintained in this laboratory. In all cases rats of comparable weights (200 to 280 gm.) born on the same day or, at most, within a day or two of each other, were employed. The mean weights of the groups within each experiment were carefully matched. The

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animals were kept in air-conditioned quarters maintained at 25.5° and 50 per cent relative humidity. They were housed in individual metabolism cages designed for the separation of urine and feces and possessing outside feeding cups. There was minimal contamination of the urine with food, feces, and drinking water. All surfaces with which the urine came into contact were covered by several coats of chemically resistant plastic paint. The cages were cleaned daily and records were kept of weights, food intake,

TABLE I  
*Composition of Diets*

In addition to the diets, each animal received daily a yeast pill (0.4 gm.) and 2 drops of propylene glycol containing 1  $\gamma$  of vitamin A alcohol and 10 U. S. P. units of vitamin D (drisdol). The sulfur-containing amino acids were added to Diets 7, 8, and 9 in quantities sufficient to bring the total sulfur supplied by amino acids approximately to that of Diet 5. The glycine added to Diet 10 supplied nitrogen equal to that of the sulfur amino acids in Diets 7, 8, and 9. The supplementary cystine and methionine in Diet 9 were added in the proportions reported for casein (9). All the diets were essentially isocaloric, containing approximately 460 calories per 100 gm. of diet.

Component	Composition of diets, gm. per 100 gm. diet									
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8	Diet 9	Diet 10
Casein (Labco vitamin-free)		5.13			18.45	55.00	5.13	5.13	5.13	5.13
Arachin			5.13	13.81						
Starch	51.30	51.30	51.30	51.30	51.30	15.10	51.30	51.30	51.30	51.30
Glucose	25.50	20.50	20.50	11.80	7.18	7.20	20.06	20.15	20.07	20.29
Lard	15.51	15.38	15.38	15.40	15.38	15.51	15.35	15.35	15.35	15.35
Cellu flour	2.06	2.06	2.06	2.06	2.06	2.06	2.06	2.06	2.06	2.06
Salt mixture (10)	5.13	5.13	5.13	5.13	5.13	5.13	5.13	5.13	5.13	5.13
Choline chloride	0.50	0.50	0.50	0.50	0.50		0.50	0.50	0.50	0.50
DL-Methionine							0.47		0.41	
L-Cystine								0.38	0.05	
Glycine										0.24

and urine volume. Urine samples were collected in a quantity of 12 per cent metaphosphoric acid adjusted so that the final concentration of the acid in the sample was between 4 and 6 per cent.

The essentially isocaloric diets employed in these studies (Table I) were supplemented with choline, vitamins A and D, and yeast. In all but one experiment 12 gm. of diet were given to each rat daily. In some experiments varying quantities of amino acids were added to the diet. After a suitable preliminary period (6 to 12 days) to insure a uniformly low pre-

experimental vitamin C excretion, the stimulating agent, sodium phenobarbital or chloretone, was administered by stomach tube or in the diet. The animals were usually fasted for 24 to 48 hours prior to the preliminary period. Occasional rats, which persisted in excreting large quantities of the vitamin prior to the administration of the drug, were discarded. It would be interesting to study such animals separately. In almost every instance all the food fed was consumed.

Determinations of 24 hour vitamin C excretions were made by the rapid indophenol titrimetric procedure in which an end-point of approximately 5 to 10 seconds was used. The results were in close agreement with those obtained by a recently developed colorimetric procedure (11). Negligible quantities of indophenol-reducing substances were found after treatment with formaldehyde in acetate buffer at pH 4 (11) in a large number of urine samples varying widely in total reducing capacity. This indicates the absence of reductones. The quantity of cysteine which was found to interfere in the determination was considerably greater than the amount likely to be found in the samples studied.

### Results

*Influence of Dosage of Phenobarbital on Vitamin C Excretion*—In the first experiment four groups of three rats each, receiving 35 ml. of evaporated milk daily, were given daily doses of 10, 20, 30, or 40 mg. of sodium phenobarbital in aqueous solution by stomach tube. The data for the last day of the control period and for the 10 days after the beginning of the administration of the drug are presented in Fig. 1. All of the animals were excreting minimal quantities of the vitamin prior to the feeding of the phenobarbital. The groups receiving the 20, 30, and 40 mg. doses gave a greater response than did the group receiving the drug at the 10 mg. level, but there were no significant differences among the excretions stimulated by the former three quantities. The 20 mg. dose was chosen for use in further experimentation, since it was the lowest dose evoking the maximal response. The gradual rise of the daily excretion of vitamin C to maximal levels is similar to that found by others (2-5).

*Influence of Dietary Level of Casein on Vitamin C Excretion*—The results of an experiment in which Diets 2, 5, and 6, containing approximately 5, 18, and 55 per cent casein, respectively, were fed to three groups of four rats each, are shown in Fig. 2. The rats were fasted for 48 hours after removal from the stock diet and then fed the evaporated milk diet for 12 days, at which time only small quantities of vitamin C were being excreted. The experimental diets were then begun. From the 4th day until the 14th after the beginning of the experimental diet all of the rats were excreting 0.2 mg. or less of vitamin C per day. On the 14th day (zero day in Fig. 2)

each rat was given 20 mg. of sodium phenobarbital dissolved in 0.5 ml. of water by stomach tube.

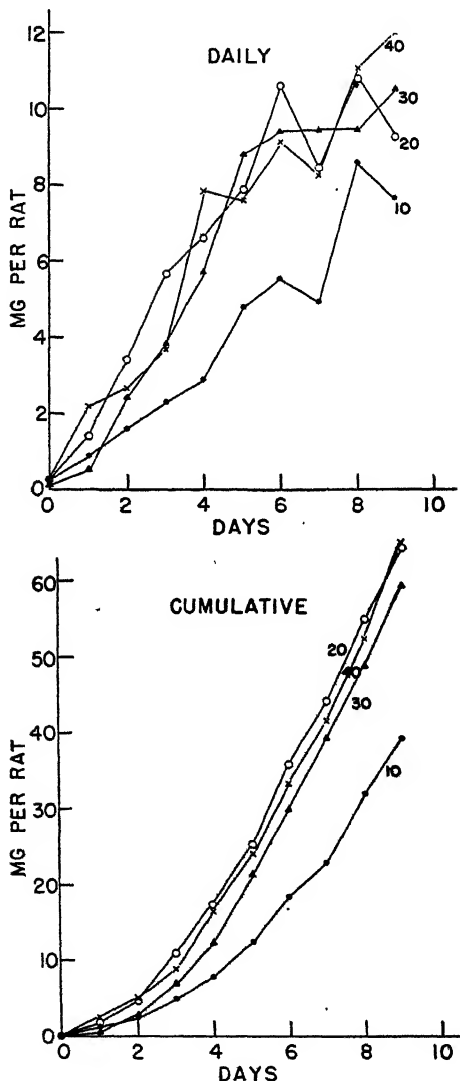


FIG. 1. Influence of dosage of phenobarbital on vitamin C excretion

The 18 per cent casein diet (Diet 5) supported the maximal vitamin C excretion, the excretion of the group receiving this diet for the 18 day experimental period being 122.5 mg. as compared to 86.5 mg. for the 55 per

cent casein diet (Diet 6) and 34.5 mg. for the 5 per cent diet (Diet 2). The excretions for the 18 and 55 per cent groups were virtually identical for the

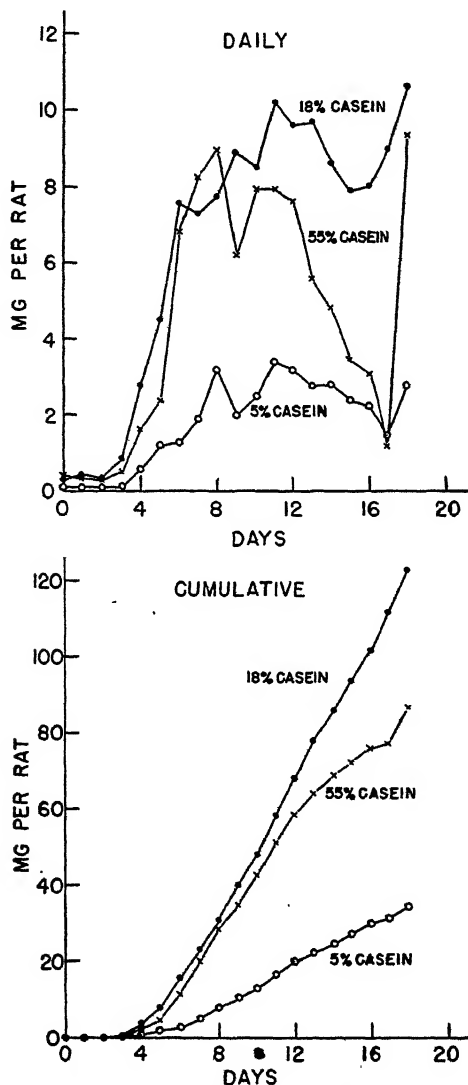


FIG. 2. Influence of dietary level of casein on vitamin C excretion of rats fed phenobarbital.

first 7 days, the 55 per cent group falling to values below that of the 18 per cent group for the remainder of the experimental period. At no time during the experiment did the mean values for the 5 and 18 per cent casein

diets overlap and on only 1 day did the value for the 55 per cent group fail to be higher than the corresponding one for the 5 per cent group. From these results it appears that both an excess and an insufficiency of dietary casein can depress the ability of the rat to excrete, and probably synthesize, vitamin C in response to a given quantity of stimulating agent.

The mean increases in weight for the period of feeding the experimental diets prior to the administration of phenobarbital were 10, 25, and 16 gm. for the 5, 18, and 55 per cent groups, respectively, and 18, 10, and 22 gm. for the period during which the phenobarbital was fed. Thus, there was no direct correlation between the changes in weight and the excretion of vitamin C. Since this was found to be the case in most of the experiments, the changes in weight in the remaining experiments will not be tabulated.

To check the above results the diets of the 5 per cent and 18 per cent casein groups were interchanged. At the completion of the first experiment the rats were placed on the evaporated milk diet for 10 days. For 12 days thereafter the group previously receiving the 5 per cent casein diet was given the 18 per cent diet and the one that had received the 18 per cent diet was placed on the 5 per cent diet without the stimulating agent. The group which had received the 55 per cent diet was given the same diet again. The administration of phenobarbital was begun on the 13th day and continued for 15 days. The results for the 9 days prior to feeding the phenobarbital and for 15 days after the first feeding of the drug are summarized in Table II. There was a marked drop in excretion in each case when the rats were changed from the 18 to the 5 per cent casein regimen, while there was an increase when the reverse procedure was applied. There was a decrease in the ability of the rats maintained on the 55 per cent casein diet to excrete vitamin C in the second experimental period. This is consistent with the decrease in excretion shown by this group of animals after the 7th experimental day in the first period (see Fig. 1). These experiments definitely establish the superiority of the 18 per cent casein diet in supporting vitamin C excretion under our experimental conditions.

*Influence of Supplementary Amino Acids on Vitamin C Excretion of Rats Fed 5 Per Cent Casein (Diet 2)*—Screening experiments were performed in which lysine, phenylalanine, threonine, tryptophan, valine, histidine, arginine, isoleucine, leucine, methionine, and cystine were added singly to the 5 per cent casein diet, in order to determine whether the difference in the effectiveness of the 5 and 18 per cent casein diets in supporting accelerated excretion of vitamin C could be ascribed to one or more of the constituent amino acids. Only cystine and methionine appeared to show a marked accelerating effect on vitamin C excretion (Table III). The group of rats receiving 100 to 200 mg. of methionine daily excreted 69.8 mg. of the vitamin over a period of 12 days as compared to 31.5 mg. for a

comparable control group, while the group receiving 100 to 200 mg. of cystine daily excreted 32.1 mg. as compared to 20.6 mg. for the controls for an 8 day period. The excretion of vitamin C by the groups receiving the sulfur-containing amino acids was higher than that of the controls for the entire period during which the amino acids were fed. How-

TABLE II

*Influence of Dietary Level of Casein on Vitamin C Excretion in Individual Rats*

Rat No.	Period No.	Diet	Total vitamin C excretion	
			Control, 9 days	Phenobarbital, 15 days
			mg.	mg.
8	1	5*	1.6	78.7
	2	2†	0.9	16.3
13	1	5	1.2	76.6
	2	2	1.0	23.2
17	1	5	1.5	134.7
	2	2	1.4	60.4
2	1	5	0.9	88.7
	2	2	0.7	52.6
9	1	2	0.3	19.3
	2	5	0.4	50.7
10	1	2	0.4	11.5
	2	5	0.4	55.4
7	1	2	0.6	49.0
	2	5	0.7	54.1
18	1	2	0.8	31.9
	2	5	0.7	90.2
11	1	6‡	1.0	64.7
	2	6	0.9	48.8
6	1	6	1.8	72.9
	2	6	1.2	55.7
15	1	6	1.7	75.0
	2	6	1.0	42.3
16	1	6	1.3	68.7
	2	6	0.9	34.2

\* 18.45 per cent casein diet.

† 5.13 per cent casein diet.

‡ 55.0 per cent casein diet.

ever, the maximal excretion was not attained for 5 to 7 days after the first daily feeding of these amino acids, although all of the rats employed had been receiving phenobarbital by stomach tube daily for at least 3 weeks. This suggests that the sulfur amino acids are necessary for some part of the mechanism by which vitamin C is synthesized in the rat, rather than



as precursors. It also seems possible that the lag between the first feeding of the stimulating agent and the attainment of maximal excretion may be dependent on the rate of synthesis of the enzymic mechanisms necessary for the synthesis of vitamin C.

*Influence of Cystine and Methionine on Vitamin C Excretion When Added to 5 Per Cent Casein Diet (Diet 2)*—Three groups of four rats each were fed (1) the 18 per cent casein diet (Diet 5), (2) the 5 per cent casein diet to which methionine and cystine had been added in the proportions found in casein (Diet 9) to bring the amino acid sulfur content up to that of the 18 per cent casein diet, or (3) the 5 per cent casein diet supplemented with glycine (Diet 10) in quantity sufficient to furnish nitrogen equal to that in the supplementary amino acids of Diet 9. Since the animals in this experi-

TABLE III

*Influence of Supplementary Methionine and Cystine on Vitamin C Excretion*

All the rats were fed 5 per cent casein (Diet 2) and had been receiving 20 mg. of sodium phenobarbital by stomach tube daily for at least 3 weeks prior to this experiment.

Supplement	No. of rats	Total excretion of vitamin C per rat		
		Days 1-3	Days 4-12	Days 1-12
		mg.	mg.	mg.
None.....	5	7.6	23.9	31.5
Methionine.....	3	9.5	60.3	69.8
		Days 1-3	Days 4-8	Days 1-8
None.....	5	8.3	12.3	20.6
Cystine.....	3	8.6	23.5	32.1

ment were somewhat heavier than those previously employed, they were given 15 gm. of food daily instead of the usual 12 gm.

The rats receiving the diet containing supplementary cystine and methionine excreted more vitamin C than did the other groups, while the rats receiving the glycine excreted the least (Fig. 3). The total excretions for the 13 days of the experiment of the groups on Diets 9, 5, and 10 were 85, 65, and 42 mg. of vitamin C, respectively. This shows clearly that the difference between the 5 and 18 per cent casein diets in supporting vitamin C excretion may be bridged by the addition of methionine and cystine to the former diet to make the total content of these amino acids in both diets approximately the same. In this experiment the excretion of the group on the 18 per cent casein diet was somewhat lower than that found previously for the same period in a similarly fed group (Fig. 1). The difference may possibly have resulted from the fact that the rats in the present ex-

periment received 3 gm. more food each day. The *total* quantity of protein consumed each day may be as important as the proportion of protein in

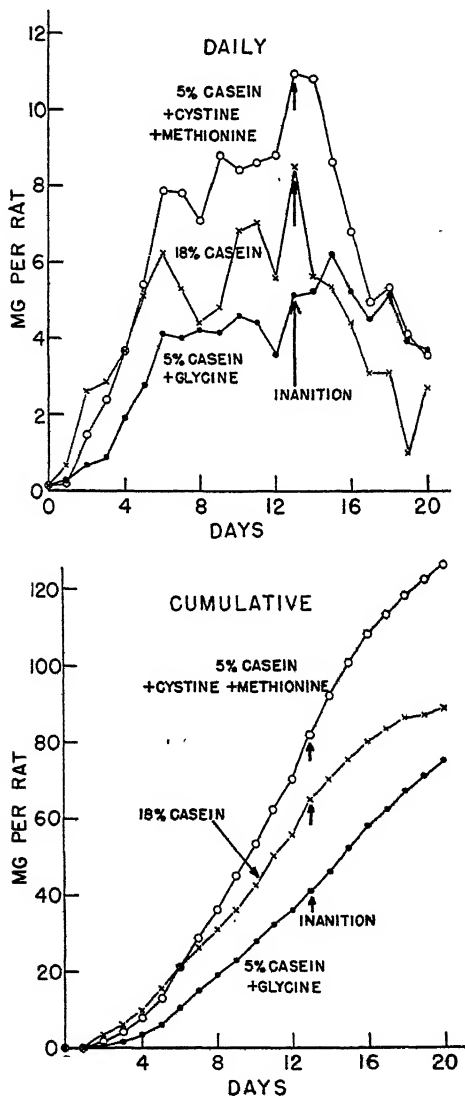


Fig. 3. Influence of the addition of cystine and methionine to the 5 per cent casein diet on the vitamin C excretion of rats receiving phenobarbital.

the diet in determining the effectiveness of a diet in supporting accelerated vitamin C excretion.

The effect of previous diet on urinary levels of vitamin C during inanition was also studied (Fig. 3). All food was removed on the 13th day after the start of the experiment, but the feeding of the phenobarbital was continued daily through the 20th day. The rats receiving Diet 10 showed a slightly greater vitamin C excretion during the 7 days of inanition than during the preceding week. The rats on Diet 9 exhibited a significant drop in excretion until the values shown by the group on Diet 10 were attained, from which time the values for the two groups were virtually identical. The group receiving Diet 5 dropped to levels lower than those of the other two experimental groups. The explanation for these findings is not readily apparent.

*Influence of Sulfur Amino Acids on Vitamin C Excretion When Added to 5 Per Cent Casein Diet (Diet 2) with Chloretone As Stimulating Agent*—Three groups of four rats each were given the 5 per cent casein diet supplemented with methionine (Diet 7), cystine (Diet 8), or glycine (Diet 10) in such a manner that the sulfur content of the first two diets was approximately equal to that of an 18 per cent casein diet (Diet 5), and the nitrogen content of all three diets was equal. Chloretone was used as the stimulating agent in this experiment, being fed in an equimolar quantity, 15 mg. per rat daily, to the phenobarbital employed in previous experiments. The drug, contained in 0.5 ml. of 95 per cent alcohol, was pipetted onto the daily food ration (12 gm. per rat) and mixed in thoroughly.

The cystine- and methionine-fed groups showed virtually identical responses, both groups excreting approximately twice as much vitamin C over the experimental period as the rats receiving glycine (Fig. 4). The average rate of excretion under these conditions was approximately the same as with phenobarbital. The results of this experiment show that the effect of the sulfur amino acids is independent of the stimulating agent and that equivalent quantities of cystine and methionine are equally effective in increasing the excretion of vitamin C when added to a low casein diet containing an adequate supply of choline.

*Influence of Dietary Level of Arachin (Protein Low in Methionine) and Supplementation with Methionine on Vitamin C Excretion*—Three groups of four rats each were fed diets containing no protein (Diet 1), 5 per cent arachin (Diet 3), and 14 per cent arachin (Diet 4). In all cases, a small amount of protein was present in the daily yeast supplement. On the 9th day after the first administration of phenobarbital and for 8 days thereafter all rats received 100 mg. of methionine in the diet daily.

Prior to the administration of methionine the vitamin C excretion increased progressively with the increasing protein content of the diet (Fig. 5). The addition of methionine to the diet raised the excretion of the group on Diet 3 to the levels previously attained by the rats on Diet 4 and

increased to a considerably smaller extent the excretion of those receiving Diet 1. On the other hand, the vitamin C content of the urines of the ani-

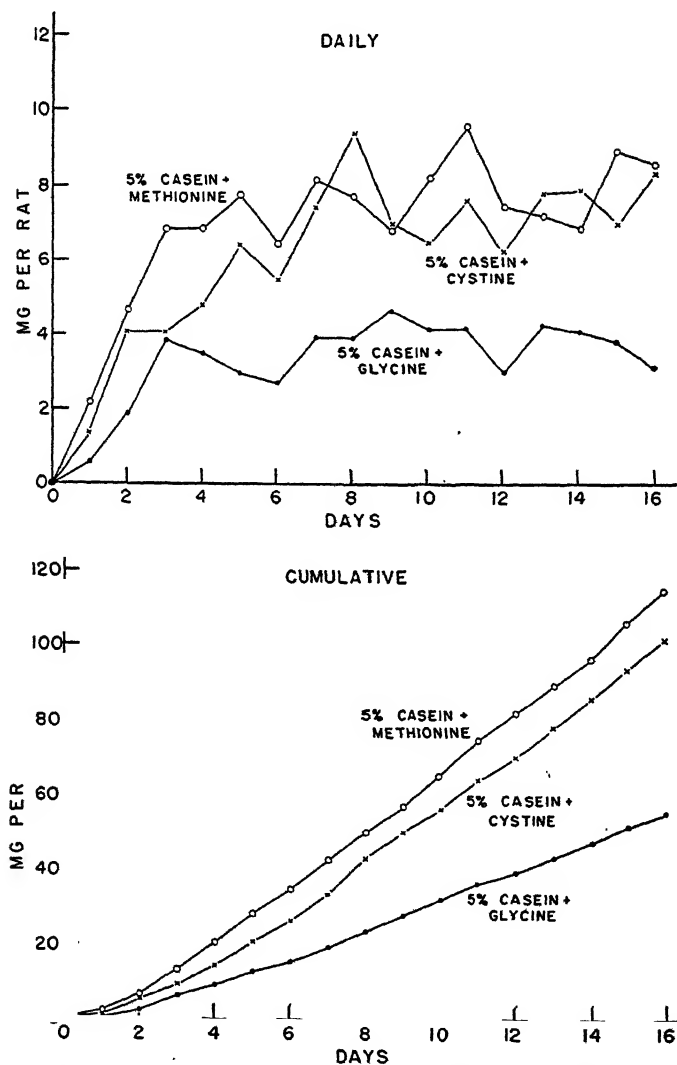


FIG. 4. Influence of the sulfur amino acids on vitamin C excretion when added to the 5 per cent casein diet with chloretone as the stimulating agent.

mals fed Diet 4 fell during the administration of methionine, until at the end of the experiment these rats were excreting vitamin C in the amounts shown by the 5 per cent arachin group prior to the addition of methionine

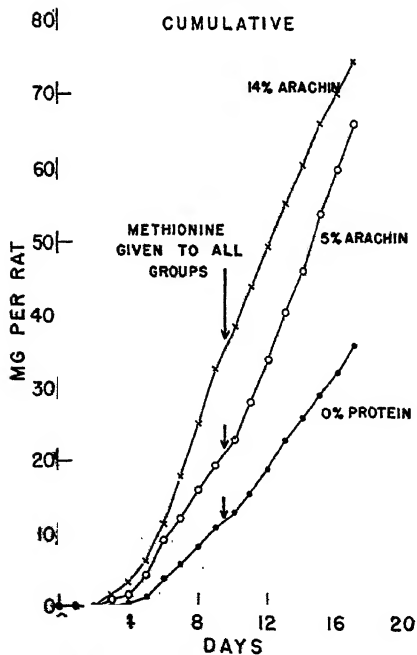
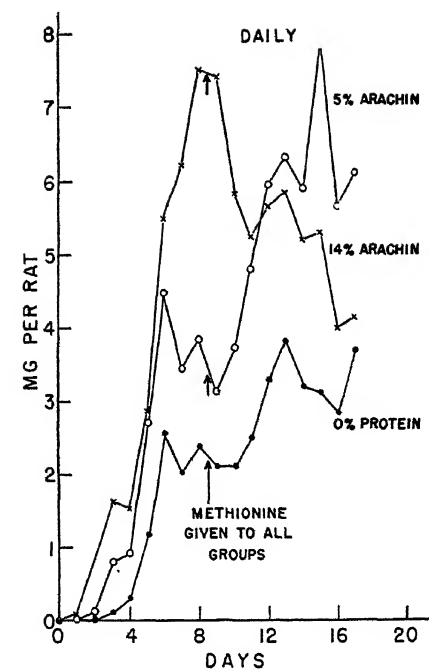


FIG. 5. Effect of dietary level of arachin and supplementation with methionine on vitamin C excretion in rats fed phenobarbital.

to the diet. Thus, depending on the arachin level of the diet, a given quantity of methionine may increase or decrease the vitamin C excretion of rats fed phenobarbital.

The total sulfur contents of Diets 1 and 3 differed only to a small extent when 100 mg. of methionine were added to these rations. However, the increase in vitamin C excretion was much greater in the group receiving 5 per cent arachin in the diet when methionine was added than in the group containing no more dietary protein than that furnished by the yeast. It appears that factors other than methionine which are present in protein are necessary for supporting the maximal vitamin C excretion and that under the conditions of Diet 1 amino acids other than methionine or cystine may be the limiting factors.

*Does Methionine Itself Stimulate Excretion of Vitamin C?*—As a result of the previously discussed experiments the question arose whether the sulfur amino acids can stimulate vitamin C excretion in the absence of other stimulating agents. Two groups of six rats each were fed 35 ml. of evaporated milk per day. On the 6th day after both groups had attained a uniformly low excretion of the vitamin, 100 mg. of methionine were added to the daily ration of one of the groups (Group A, Fig. 6). For 7 days after the addition of the methionine there was no evidence whatsoever of a stimulation of vitamin C excretion in the rats receiving the amino acid, the excretion for both groups remaining identical for this period. On the 8th day after the start of the methionine and daily for the remainder of the experiment 20 mg. of phenobarbital were given by stomach tube to each rat in both groups. The accelerated excretion of the vitamin induced by phenobarbital was not enhanced by the addition of methionine, but instead appears to have been somewhat depressed. The removal of methionine from the diet of Group A on the 24th day of the experiment did not alter the general level of excretion of these rats.

These data prove that methionine itself does not have a stimulatory effect on vitamin C excretion in the sense that phenobarbital and chloretone do, and show that when this amino acid is added to a diet which can support a high vitamin C excretion it may depress the ability to excrete vitamin C.

*Influence of Fasting on Response of Rats to Phenobarbital*—Experiments have been reported in which chloretone-fed rats excreting large quantities of vitamin C were fasted for 5 days while still receiving the drug (5). The average excretion of these animals was reduced to 2 mg. of vitamin C per day. The experiment to be described was performed to determine whether rats severely depleted by fasting could still respond to the feeding of phenobarbital with an accelerated excretion of vitamin C (Fig. 7). Twelve rats were fed 35 ml. of milk per day until a uniformly low excretion was attained and then were fasted for 3 days prior to the daily administration of 20 mg.

of phenobarbital by stomach tube. On the last day before the administration of the drug the average daily excretion was 0.1 mg. of vitamin C per

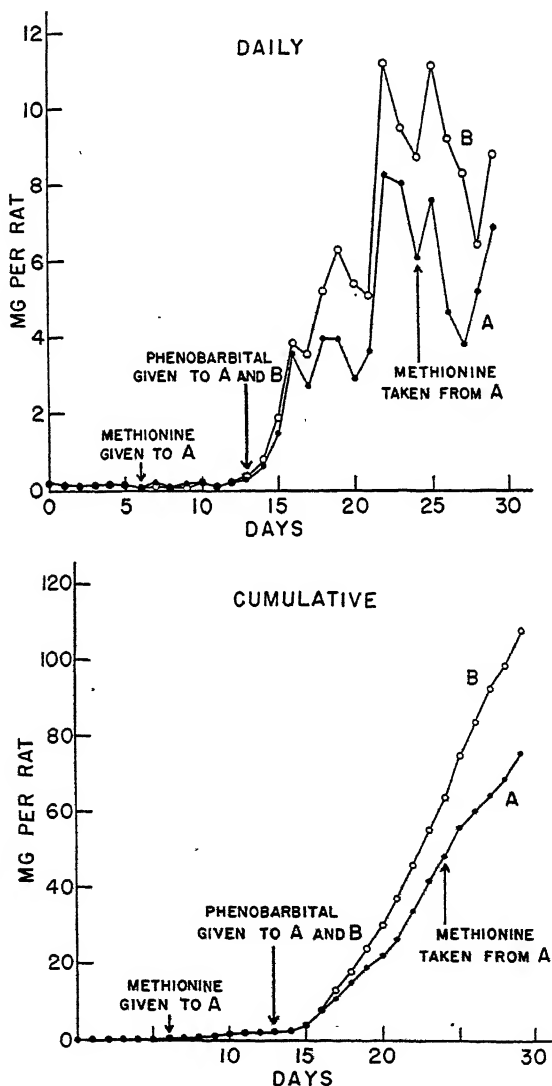


FIG. 6. Effect of methionine on vitamin C excretion before and after administration of phenobarbital in rats receiving an evaporated milk diet.

rat. There was a gradual increase in the daily excretion for 6 days and a slight drop on the 7th day after the first feeding of phenobarbital. The

mean maximal excretion of 1.1 mg. per rat on the 6th day was approximately one-tenth that shown by rats on a diet supporting a high level of vitamin C excretion. In spite of the elimination of all exogenous sources of the precursors for vitamin C and for the enzymes participating in the synthesis, the rats were able to increase the excretion 10-fold over the control value. The mean loss in weight of the rats during the period of feeding the drug was 24 per cent of the original body weight.

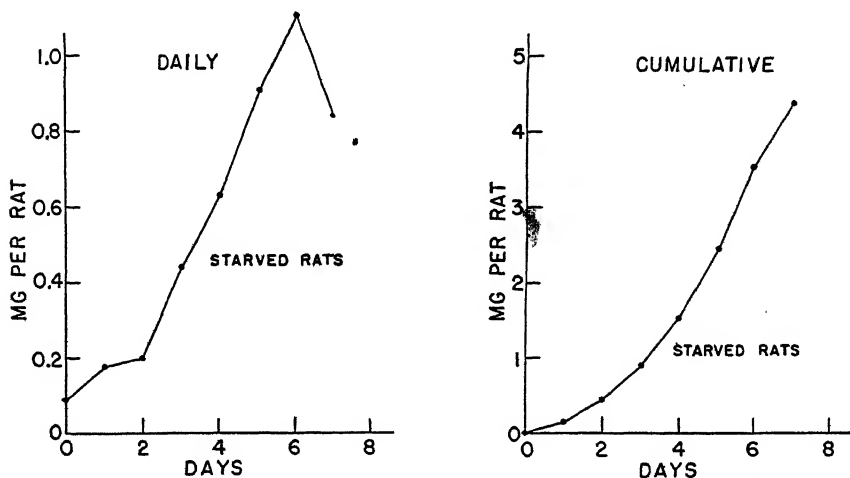


FIG. 7. Influence of fasting on the response of rats to phenobarbital

#### DISCUSSION

It has been shown that chloretone and phenobarbital not only greatly accelerate the urinary ascorbic acid excretion in rats, but also cause an increased concentration of ascorbic acid in the small intestine, kidneys, and liver and to a smaller extent in the spleen (5). Only slight changes were observed in the brain and adrenals. Tissues from chloretone-fed rats showed evidence of synthesis of vitamin C *in vitro* with a substrate mixture of pyruvate, glyceric aldehyde, and hexose diphosphate, while the tissues from normal rats showed virtually no evidence of synthesis of the vitamin from these substances (12). The former study (5) and work previously cited (2-4) show conclusively that the increased urinary excretion of vitamin C in rats fed these drugs is a result of an increased rate of synthesis. The latter paper (12) indicates that the tissues of chloretone-stimulated animals probably contain more of the enzymatic mechanisms (or activity) required in the synthesis of vitamin C. The fact that the maximal urinary excretion of vitamin C in response to a given dose of drug occurs only after a period of time (4 to 7 days) following the first feeding of the drug also



suggests that during this period there is an increased synthesis or activation of the mechanism by which the synthesis of the vitamin is achieved.

The experiments reported in this paper show that feeding a diet containing 5 per cent casein markedly limits the ability of the rat to excrete vitamin C in response to either phenobarbital or chloretone as compared with an isocaloric diet containing 18 per cent casein. The animals on both diets showed a gain in weight during the experiment. The addition of cystine or methionine or a combination of the two to the 5 per cent casein diet in quantities supplying sulfur equal to that in the 18 per cent casein diet brought the excretion up to the level supported by the latter diet, while none of the other nine essential amino acids appeared to have a similar effect. The effect of methionine in supporting enhanced vitamin C excretion was further shown in the experiment in which supplementation of a 5 per cent arachin diet with 100 mg. of methionine daily raised the excretion to levels attained with a 14 per cent arachin diet.

Although the possibility that the S-containing amino acids can serve as precursors for vitamin C has not been unequivocally ruled out, it is doubtful that this is the case. The addition of a relatively large quantity of methionine to diets containing 5 per cent arachin or casein was much more effective in increasing vitamin C excretion in rats receiving the stimulating drugs than was the addition of the methionine to an isocaloric diet containing no protein other than the yeast supplement. This indicates that the absence of other amino acids may also limit the rate of synthesis of the vitamin by the rat. The dietary conditions employed in the present experiments were especially favorable for the demonstration of the effects of cystine and methionine. Methionine was shown to have no stimulating action of its own on vitamin C excretion in rats not receiving phenobarbital or chloretone, and it was proved that the effect of the sulfur amino acid supplements was not attributable to their nitrogen content.

The type of results obtained in these studies is not unique, since many reports have been made of the beneficial effects of the supplementation of a low protein diet with the S-amino acids. A number of such studies are quoted by Miller (13), who showed clearly that L-cystine and DL-methionine have a definite protein-sparing action when fed to dogs on a very low protein diet. Croft and Peters (14) found that losses of urinary nitrogen in burned rats receiving a 10 per cent casein diet could be specifically decreased by the addition of methionine to the diet. The addition of methionine or cystine to a diet containing an inadequate amount of casein increased the regeneration of liver protein after fasting (15).

The decrease in the ability to excrete vitamin C produced by the feeding of a 55 per cent casein diet or by the addition of methionine to a 14 per cent arachin diet or an evaporated milk diet is difficult to explain, except pos-

sibly in terms of a competition for tissue protein by the enzymes of vitamin C synthesis and the catabolic enzymes involved in degrading a dietary excess of protein or amino acids.

#### SUMMARY

1. A study was made of the influence of altering the dietary level of protein on the accelerated urinary excretion of vitamin C of rats fed sodium phenobarbital or chloretone.

2. Increasing the level of casein from 5 to 18 per cent resulted in an increased ability of the rats to excrete vitamin C when fed phenobarbital. A further increase to 55 per cent resulted in a somewhat lower excretion.

3. Increasing the dietary level of arachin from 5 to 14 per cent also resulted in accelerated excretion of the vitamin.

4. Only cystine and methionine showed a marked accelerating effect on vitamin C excretion in rats fed phenobarbital in experiments in which each of the ten essential amino acids and cystine was added singly to the 5 per cent casein diet.

5. Cystine and methionine, when added to the 5 per cent casein diet to bring the total sulfur content up to that of the 18 per cent casein diet, increased the excretion of vitamin C with *either* chloretone or phenobarbital to approximately the maximal level attainable under the experimental conditions employed.

6. Methionine had no effect on vitamin C excretion in the absence of a stimulating agent.

7. The addition of methionine to a milk diet supporting a high level of vitamin C excretion depressed the excretion. A similar result was obtained when methionine was added to a 14 per cent arachin diet.

8. It is suggested that cystine and methionine probably do not act directly as precursors of vitamin C in the rat and that the accelerating influence on vitamin C excretion observed in these studies is related to the generally beneficial effects observed when these amino acids are added to a low protein diet.

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# THE COLORIMETRIC DETERMINATION OF HEXOSES WITH CARBAZOLE

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The difficulty of applying classical procedures to the qualitative and quantitative determination of carbohydrate in certain biological materials has led to the development of colorimetric methods in which the carbohydrate-containing material is treated with strong mineral acids, causing the formation of substances which will react with compounds such as diphenylamine, resorcinol, orcinol, indole, carbazole, etc., to give distinctively colored products. The rate at which these colored products are formed (1) and the nature of their absorption spectra are frequently sufficiently distinctive to allow their use in differentiating the various sugars.

One of the most widely used of the above colorimetric methods is the carbazole-sulfuric acid method first described by Dische (2-4) and further developed by Gurin and Hood (5, 6) for the identification and estimation of hexoses and pentoses. The latter procedure was used by Seibert and Atno (7) for the analysis of the polysaccharides present in serum and by Knight (8) for the identification of the sugars present in influenza virus. Dische (9) has recently described a modification of the carbazole method for the analysis of uronic acids.

Although the carbazole-sulfuric acid method has been used extensively, no systematic study of the variables influencing this method appears to have been reported. Difficulties encountered in the quantitative application of the carbazole method have been commented upon (7) and indeed the significance of the carbazole reaction, or other color tests, for the qualitative identification of sugars has been questioned (10). Recently in the course of a study of the polysaccharide fractions from hog gastric mucin, we have had occasion to investigate the more important variables associated with the carbazole reaction as applied to the determination of hexoses. In the course of this study the optimum conditions for the quantitative determination of hexose were determined and certain aspects of the qualitative identification of hexoses were examined.

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† Contribution No. 1137.

## EXPERIMENTAL

*Reagents*—Eastman White Label carbazole was precipitated three times from a concentrated sulfuric acid solution by dilution with cold water, and the dried product recrystallized from toluene. The sugars were recrystallized from aqueous ethanol according to conventional methods (11). Technical furfural was fractionally distilled and the fraction boiling at 80–81° and 50 mm. reserved for use. In order to obtain lower blanks, c.p. sulfuric acid was refluxed with potassium persulfate (20 mg. per liter) until a negative test for oxidizing agents was obtained with starch-iodide.

*Apparatus*—A Klett colorimeter, green filter No. 54, was used for all colorimetric analyses. Duplicate or triplicate analyses were always performed and the results reported are average values. A Beckman model DU spectrophotometer, equipped with a 1 cm. cell, was used in determining spectral absorption. Intensities were measured at 10  $m\mu$  intervals except

TABLE I  
*Effect of Sulfuric Acid Concentration*

Concentrated H <sub>2</sub> SO <sub>4</sub> added per cent by weight	Klett value*
80	215
82	230
84	230
	220
	180

\* Corrected for blank on 1 ml. of water.

in the region of maxima and minima where the interval was reduced to 2 to 5  $m\mu$ .

*Effect of Sulfuric Acid Concentration*—With the amount of carbazole set at 1.5 mg., the quantity of hexose at 100  $\gamma$  of glucose per ml., and 10 minutes for the time of heating, variation of the sulfuric acid concentration gave rise to the values shown in Table I. Similar results were obtained with galactose.

*Effect of Carbazole Concentration*—With the concentration of the added sulfuric acid maintained at 84 per cent (by weight), the amount of hexose at 100  $\gamma$  of glucose per ml., and the time of heating at 10 minutes, variation of the carbazole concentration between the limits of 1.5 and 4.5 mg. gave rise to the values presented in Table II. Similar results were obtained with galactose.

*Effect of Time of Heating*—Test solutions containing 9 ml. of 84 per cent

sulfuric acid, 3 mg. of carbazole, and 100  $\gamma$  of glucose in 1 ml. of water were heated in a boiling water bath for varying periods. It was found that 80 per cent of the maximum color intensity was attained after heating for 7½ minutes, 99 per cent after 10 minutes, 100 per cent after 15 minutes, and 97 per cent after 20 minutes. As in other experiments galactose gave similar results.

TABLE II  
*Effect of Carbazole Concentration*

Carbazole present	Klett value*
mg.	
1.5	240
2.5	316
3.0	363
3.5	402
4.5	438

\* Corrected for blank on 1 ml. of water.

TABLE III  
*Determination of Glucose with Modified Procedure*

Glucose	Klett value*	Average deviation, Klett units
$\gamma$		
140	682	6
120	607	12
100	527	11
80	425	10
60	349	3
40	272	4
20	185	7
10	144	4
5	129	5

\* Average of six separate determinations.

*Modified Procedure*<sup>1</sup>—A reagent was prepared by adding 10 ml. of a 1.0 per cent solution of carbazole in absolute ethanol to 300 ml. of 84 per cent sulfuric acid. 9 ml. portions of this reagent were chilled in an ice bath, 1 ml. of the hexose solution poured onto the reagent, and the solutions

<sup>1</sup> The procedure of Gurin and Hood (5) consists of heating 1.5 mg. of carbazole (0.3 ml. of a 0.5 per cent solution in ethanol), 1 ml. of hexose solution, and 9 ml. of 89 per cent sulfuric acid (8:1 concentrated sulfuric acid and water) for 10 minutes in a boiling water bath.

thoroughly mixed and heated in a boiling water bath for 15 minutes. After cooling in an ice bath, the intensity of the color produced was determined in the Klett colorimeter. Typical results, obtained with glucose, are given in Table III.

#### DISCUSSION

It is seen from Table I that the Klett values are particularly dependent upon the acid concentration and that maximum color intensity is obtained with 82 to 84 per cent added sulfuric acid. It was shown, by means of extinction curves, that the differences noted in Table I were due to differences in intensity alone. While extinction values varied widely with sulfuric acid concentration, the position of the maxima lay between 540 and 550  $m\mu$  in every instance. However, it should be pointed out that qualitative observations of Dische (2) suggest that significant changes in respect to the position of the maxima may occur also if the sulfuric acid concentration is varied widely.

The dependence of the Klett values upon the carbazole concentration (Table II) emphasizes the necessity of precision in adding the carbazole to the reaction mixture. An error of 3 per cent in the addition of 1.5 mg. of carbazole (0.3 ml. of a 0.5 per cent solution in ethanol) would cause a corresponding variation of about 4 units in Klett values. It is obvious that the addition of small volumes of a carbazole solution is undesirable, especially since the solvent is ordinarily absolute ethanol, which is difficult to pipette accurately. While large amounts of carbazole undoubtedly increase the sensitivity of the procedure, especially since the blank values are practically constant over the range studied, the low solubility of carbazole in the diluted sulfuric acid limits the upper concentration.

In contrast to other variables the time of heating is not particularly critical, provided the time is not less than 10 minutes or more than 20 minutes. A period of heating of 15 minutes, with 84 per cent sulfuric acid, appears to be a reasonable choice.

In order to simplify the procedure of Gurin and Hood (5) the carbazole was dissolved in a relatively large quantity of 84 per cent sulfuric acid and aliquots of this reagent were used for analysis. This reagent simplified the procedure for routine analysis as well as eliminated errors arising from the addition of carbazole to individual tubes. The reagent is prepared by mixing an ethanolic solution of carbazole with 84 per cent sulfuric acid, because solid carbazole dissolves very slowly in sulfuric acid of this concentration. Although the reagent is known to be stable for at least 6 hours, occasionally a green color has appeared after standing for more than 24 hours. It is recommended that the reagent be prepared daily, as was suggested by Seibert and Atno (7).

Nitrate and ferric iron are presumed to interfere in the carbazole procedure of Gurin and Hood (5). No interference from these constituents, or from nitrite, was observed with the modified procedure. The same Klett values were obtained, within the limits of precision discussed below, in the presence or absence of 5  $\gamma$  of sodium nitrate, sodium nitrite, or ferric chloride when the amount of hexose present was 100  $\gamma$  of glucose. Sodium

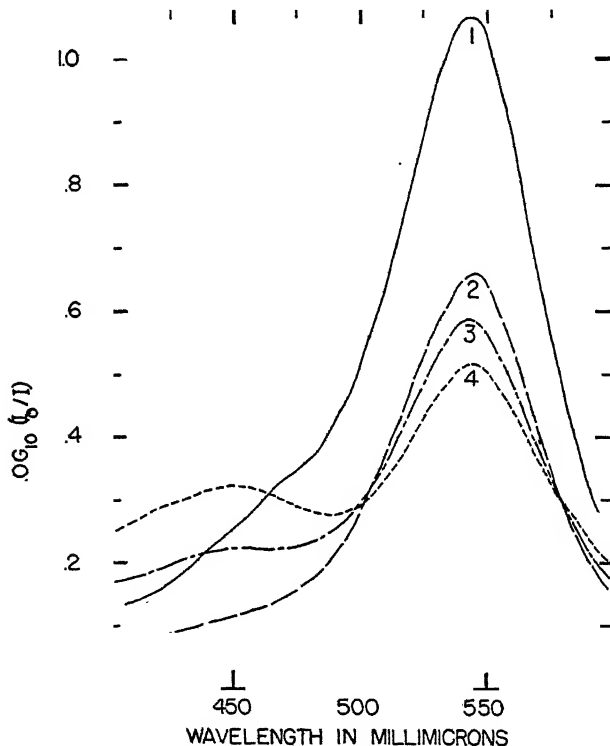


FIG. 1. Absorption curves for colors obtained with various sugars on reaction with carbazole. Curve 1, 100  $\gamma$  of fructose; Curve 2, 100  $\gamma$  of glucose; Curve 3, 100  $\gamma$  of galactose; Curve 4, 100  $\gamma$  of mannose.

nitrite imparted a faint green color, as did ferric chloride at higher concentrations, to the cold carbazole-sulfuric acid solution. However, this color generally disappears on heating.

While the modified procedure possesses the advantages of convenience and reliability, the precision would appear to be no greater than that of the original when the latter is applied with extreme care. The modified procedure has a precision of 2 to 5 per cent (Table III) in the range of 50 to 150



$\gamma$  of glucose. The relatively low precision is still unexplained, although the complexity of the reactions occurring in sulfuric acid, revealed by the extinction curves discussed below, suggests that experimental conditions may not still be sufficiently reproducible.

The extinction curves for the carbazole-hexose colored products obtained by the modified procedure are shown in Fig. 1. The curves resemble qualitatively those obtained previously (7, 8). However, the color ob-

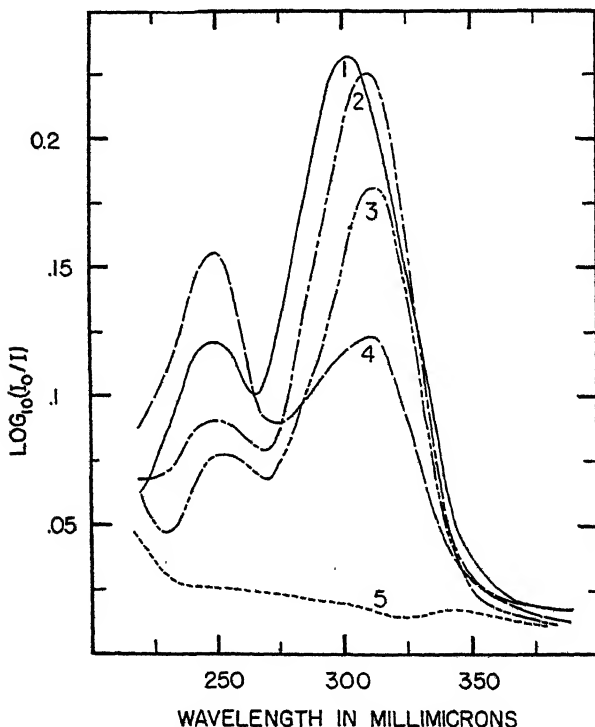


FIG. 2. Absorption curves for various sugars heated in sulfuric acid solution. Curve 1, 100  $\gamma$  of galactose; Curve 2, 100  $\gamma$  of glucose; Curve 3, 100  $\gamma$  of fructose; Curve 4, 100  $\gamma$  of mannose; Curve 5, 100  $\gamma$  of N-acetylglucosamine.

tained with mannose does not appear to be as markedly different from that of the other hexoses as has been observed by others (7, 8). It would appear that precise control of the sulfuric acid concentration is of utmost importance, not only for quantitative procedures, but also in the qualitative interpretation of absorption spectra. Owing to the similarity of the curves for glucose, fructose, mannose, and galactose, the low precision of the carbazole method, and because of possible interferences from other

types of compounds (5), the qualitative identification of sugars by means of the modified procedure would appear to be dubious.

Relevant to the problem of spectral identification of sugars are the extinction curves in the ultraviolet region that have been obtained for sugars in sulfuric acid solution in the absence of carbazole. Fig. 2 shows curves obtained for 100  $\gamma$  of sugar in 1 ml. of water and 9 ml. of 84 per cent sulfuric acid solution after heating 15 minutes on the water bath. The hex-

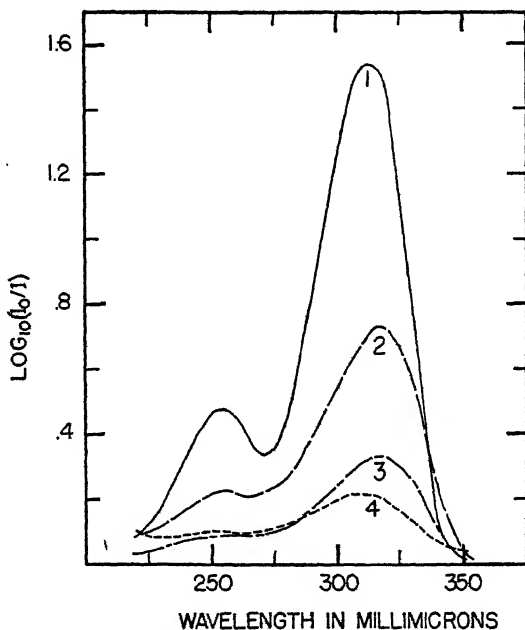


FIG. 3. Absorption curves for furfural and various sugars in sulfuric acid solution. Curve 1, 100  $\gamma$  of furfural; Curve 2, 100  $\gamma$  of fructose after standing 40 minutes; Curve 3, 100  $\gamma$  of fructose after standing 5 minutes; Curve 4, 100  $\gamma$  of fructose after standing 40 minutes and then heating 15 minutes in a water bath. Curves for galactose, mannose, glucose, and N-acetylglucosamine are not shown, since the densities are below 0.03 throughout the wave-length range.

oses studied exhibit maxima at about 250 and 320  $m\mu$ . Fructose, glucose, and galactose show similar curves, while mannose differs in having less absorption at 320  $m\mu$  than at 250  $m\mu$ . The positions of the maxima correspond closely to those observed for furfural under the same conditions; the apparent conversion of the hexoses to a furfural derivative would appear to be only 10 to 20 per cent as judged from the spectra. While the mechanism of the carbazole reaction and similar color tests is not clearly

understood, presumably the formation of aldehyde intermediates is important (2, 9). It is significant that N-acetylglucosamine, which does not give a color test with carbazole, also shows no specific absorption in the ultraviolet in sulfuric acid solution.

The relative heights of the maxima in the ultraviolet are not correlated with the intensity of the colors produced in the carbazole reaction, a fact which might argue for the unimportance of the compounds showing ultraviolet absorption in the subsequent color reaction. However, extinction curves of unheated sulfuric acid solutions of the hexoses (Fig. 3) suggest a complicating feature. The ultraviolet spectra were found to be sensitive to time of heating, and the extinction values increased and then decreased on heating for successively longer periods. Glucose, fructose, mannose, and N-acetylglucosamine show no appreciable absorption in the cold, while fructose is converted rapidly to an intermediate showing specific absorption in the region 250 to 320  $m\mu$ ; maximum absorption is reached after about 40 minutes at 25°. When the fructose solution is then heated, the specific absorption decreases markedly, indicating other reactions leading to decomposition. Fructose also shows characteristic behavior in the carbazole reaction in that a color appears several times faster and with greater intensity than for any of the other hexoses. It is apparent that the marked difference in behavior of fructose from other sugars in cold sulfuric acid could readily be adapted to its detection under suitable conditions. Since it is known that heated acid solutions of the hexoses will not react appreciably with carbazole in the cold (9), the carbazole reaction would appear to consist of at least two series of reactions, (1) the conversion of hexoses to intermediates showing specific ultraviolet absorption and the simultaneous decomposition of these intermediates in hot acid solution, and (2) the reaction of some or all of the products with carbazole in hot acid solution to yield a stable visible color.

#### SUMMARY

The optimum conditions for the colorimetric estimation of hexoses by reaction with carbazole in hot sulfuric acid solution have been determined and a convenient procedure, giving results with a precision of 2 to 5 per cent in the range of 50 to 150  $\gamma$  of glucose, is described. The colors obtained with glucose, galactose, fructose, and mannose are not sufficiently distinctive to allow their ready differentiation and identification by spectral measurements. The significance of the ultraviolet spectra of heated and unheated sulfuric acid solutions of hexoses to the problem of estimation and identification of hexoses is discussed.

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# THE RÔLE OF PROLINE, HYDROXYPROLINE, AND GLUTAMIC ACID IN GROWTH\*

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In a recent paper from this laboratory (1) arginine was shown to be a necessary dietary component of the rat for *optimum* growth. Upon rations of otherwise high quality young animals deprived of arginine gained little more than half as much as their litter mates which received this amino acid. These findings were interpreted as indicating that, in the species in question, the synthesis of arginine does not keep pace with the requirements of the cells for *maximum* increases in weight.

As pointed out in the above paper, the question of the growth effects of glutamic acid and the prolines still remains to be answered. Several years ago an attempt was made to solve this problem, as well as the closely related one of the possible interchangeability of these amino acids and arginine in metabolism, by removing all four compounds from hydrolyzed casein (2). The results were inconclusive inasmuch as no method existed whereby the completeness of the removal could be ascertained. Following the discovery of threonine (3), the feasibility of formulating rations containing known mixtures of the amino acids in place of proteins again directed our attention to the rôle of glutamic acid and the prolines. Preliminary experiments, in which the influence of each was compared to that of arginine, yielded what appeared to be essentially negative results. Of the three amino acids, proline alone seemed to exert a slight growth-stimulatory action, but this was so small as to be regarded as insignificant.

Without attempting to justify this conclusion, which admittedly was scarcely warranted by the relatively small number of animals employed in the preliminary tests, it is pertinent to point out that all such feeding trials were handicapped at the time by the want of a suitable source of vitamins. Under the most favorable circumstances then possible, animals receiving so called "synthetic" rations manifested gains which would now be regarded as distinctly subnormal. Even the stimulatory action of arginine was found to be relatively small until slowly acquired information with respect to the dietary requirements of the rat, and the advent of adequate supplies

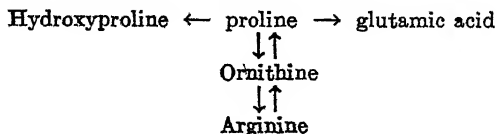
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of crystalline vitamins, permitted a thorough overhauling of our basal ration (*cf.* Borman *et al.* (1)).

The above facts called for a reinvestigation of the growth effects of glutamic acid and the prolines. Furthermore, this need was rendered more insistent by evidence appearing in the literature to the effect that certain interconversions of the 5-carbon amino acids actually occur. Thus, Weil-Malherbe and Krebs (4) succeeded in demonstrating the formation of  $\alpha$ -ketoglutaric acid by the action of kidney slices upon proline. In similar experiments involving the use of liver slices, Neber (5) isolated glutamic acid. In the presence of ammonia kidney slices transform proline and hydroxyproline into amide nitrogen (glutamine) (4, 5). Hydroxyproline is said to be less effective than proline in this respect, nor does it yield an appreciable amount of  $\alpha$ -ketoglutaric acid (4). Subsequently, Krebs showed that mammalian kidney extracts transform both D-proline and D-ornithine into  $\alpha$ -keto- $\delta$ -aminovaleric acid (6). The latter acid appears to be produced also by the action of L-amino acid oxidase upon L-proline (7). These facts suggest the possible interconversion of proline and ornithine, although the mechanism may not be so simple as the discovery of the above intermediate seemed to imply (*cf.* Shemin and Rittenberg (8)).

It remained for the Columbia University group, by the use of amino acids "labeled" with isotopes, to demonstrate that these and other interconversions involving the 5-carbon amino acids occur in the intact animal. In a brilliantly conceived and skilfully executed series of investigations Schoenheimer and his associates proved that deuterioornithine, in the organism of the mouse, is not only converted into deuterioarginine (9), but into deuteroproline and deuteroglutaric acid as well (10). Furthermore, proline containing both deuterium and  $N^{15}$  is transformed by the rat into isotopic hydroxyproline, arginine, and glutamic acid (11). This shows that proline and ornithine (arginine) are mutually interconvertible *in vivo*. On the other hand, there is as yet no conclusive evidence for the conversion of hydroxyproline or glutamic acid into proline in the intact animal. The over-all relationships thus far established, hypothetical intermediates being omitted (*cf.* (11, 8)), are indicated below.



While no reasonable doubt exists that the transformations indicated by the arrows in the above scheme actually occur *in vivo*, it does not follow necessarily that the *rates* of interconversion are sufficiently rapid to meet the demands of *normal* growth. If proline and arginine are interconverti-

ble with sufficient speed, the two amino acids should be mutually interchangeable in the diet. If the formation of hydroxyproline and glutamic acid from proline or ornithine represents reversible reactions, either of the former should be capable of replacing arginine of the food to the extent that the rates of transformation keep pace with the needs of the cells. If all four amino acids are interconvertible at velocities equal to the tissue demand, the presence of any one in the diet should be as effective upon growth as when all are supplied preformed. Such were the considerations which motivated us in carrying out the feeding trials described in this paper.

#### EXPERIMENTAL

Male weanling rats served as the experimental subjects throughout. Each animal was housed in a separate cage and was permitted to consume the diet *ad libitum*. Each test was continued for 28 days. Amino acids furnished the nitrogen of the rations and were purified invariably until they yielded correct analytical values.

The composition of the amino acid mixture (Mixture XXIII-b) is shown in Table I. As will be observed, it was devoid of arginine, the prolines, and glutamic acid. These were incorporated in the diets separately as indicated below. The make-up of the basal diet is presented in Table II. The unknown nitrogen present in the liver extract did not exceed 32 mg. per 100 gm. of food, and consequently could not have contributed significant amounts of the amino acids under investigation. In comparing the growth effects of proline, hydroxyproline, and glutamic acid with that induced by arginine, the desired amount of each amino acid was added to the basal diet in place of an equal weight of dextrin. In certain tests the nitrogen content of all rations was equalized. In that event, the nitrogen present in the negative control diet was raised by an appropriate increase in the level of Mixture XXIII-b at the expense of the dextrin. In every experiment, each kilo of ration was supplemented with vitamins by the thorough admixture of the quantities listed in Table III.

Table IV summarizes the comparative growth effects of the four amino acids when incorporated *singly* in the basal ration. Throughout the experiments recorded in Table IV the total nitrogen content of the diets being compared was the same except in the glutamic acid tests of Series I. In this test, for reasons which need not be discussed at this time, nitrogen was furnished at a slightly (0.025 per cent) lower level. This fact did not affect the outcome of the tests, as will be explained later. Since Series I involved a comparison of five diets, and since male animals only were employed, it was not feasible to distribute the members of each litter among all of the test rations. The 121 rats used in this series were derived from



TABLE I  
*Composition of Amino Acid Mixture*

	Mixture XXIII-b	
	Physiologically active	As used
	gm.	gm.
Glycine.....	0.10	0.10
Alanine.....	0.20	0.40*
Serine.....	0.10	0.20*
Valine.....	1.00	2.00*
Leucine.....	1.20	2.40*
Isoleucine.....	0.80	1.60*
Cystine.....	0.20	0.20
Methionine.....	0.80	0.80*
Threonine.....	0.70	1.40*
Phenylalanine.....	1.20	1.20*
Tyrosine.....	0.60	0.60
Tryptophan.....	0.40	0.40*
Aspartic acid.....	0.20	0.40*
Lysine.....	1.20	
“ monohydrochloride.....		1.50
Histidine.....	0.70	
“ monohydrochloride monohydrate.....		0.95
Sodium bicarbonate.....		1.07
	9.40	15.22

\* Racemic acids. In certain of the tests the DL-leucine was replaced by half its weight of L-leucine. This did not induce a detectable difference in growth rate. In one series of experiments the DL-tryptophan was replaced by an equal weight of L-tryptophan. This will be referred to later.

TABLE II  
*Composition of Basal Diet*

	gm.
Amino acid Mixture XXIII-b.....	15.22
Sucrose.....	15.00
Dextrin.....	61.03
Cellu flour.....	2.00
Salt mixture*.....	4.00
Corn oil.....	2.00
Vitamin A and D concentrate†.....	0.05
Inositol.....	0.10
Choline chloride.....	0.20
Liver extract‡.....	0.40
	100.00

\* Jones and Foster (12).

† This contained 65,000 U. S. P. units of vitamin A and 13,000 U. S. P. units of vitamin D per gm.

‡ Wilson's liver powder 1:20. Grateful acknowledgment is made to Dr. David Klein of The Wilson Laboratories for his generosity in furnishing this product.

twenty-five litters. This relatively large number of animals should minimize the influence of any inherent differences in growth tendency of individual litters. In Series II and III, each involving a comparison of only two diets, litter mates were used invariably.

TABLE III  
*Vitamin Supplements*

	Added to each kilo of diet
	mg.
Thiamine hydrochloride.....	5
Riboflavin.....	10
Pyridoxine hydrochloride.....	5
Nicotinic acid.....	5
Calcium <i>d</i> -pantothenate.....	25
<i>p</i> -Aminobenzoic acid.....	300
$\alpha$ -Tocopherol.....	25
2-Methyl-1,4-naphthoquinone.....	2
	$\gamma$
Biotin.....	100

TABLE IV  
*Comparative Growth Effects of Arginine, Proline, Hydroxyproline, and Glutamic Acid\**  
The experiments covered 28 days each.

Series No.	Supplement	Amount in diet	No. of animals	Mean gain in weight and probable error of mean
		per cent		gm.
I	None		30	49.1 $\pm$ 0.85
	Arginine	1.13†	31	78.2 $\pm$ 1.25
	Proline	3.00	28	60.0 $\pm$ 1.26
	Hydroxyproline	3.41	9	26.2 $\pm$ 1.70
	Glutamic acid	3.58	23	58.0 $\pm$ 1.10
II	None		7	48.0 $\pm$ 0.53
	Glutamic acid	3.84	12	56.7 $\pm$ 1.26
III	None		10	48.2 $\pm$ 0.96
	Hydroxyproline	1.00	10	43.1 $\pm$ 0.92

\* Each supplementary amino acid possessed the L configuration.

† Administered as the monohydrochloride (1.37 per cent of the diet) with an equivalent amount of sodium bicarbonate.

The results demonstrate that, contrary to our unpublished findings of several years ago, involving the use of a less satisfactory basal diet, both proline and glutamic acid exert statistically significant effects upon the growth of rats deprived of arginine. In the glutamic acid tests the growth

acceleration was comparable whether the amino acid was furnished at a level of 3.84 per cent of the diet (Series II), equivalent to the nitrogen in 1.13 gm. of arginine, or at the slightly lower level of 3.58 per cent (Series I). On the other hand, the data demonstrate quite clearly that neither proline nor glutamic acid is as effective a growth stimulant as is arginine. The mean growth increment induced by arginine was 29.1 gm. The corresponding figures for proline and glutamic acid were 10.9 and 8.9 gm., respectively. These differences become even more striking when one recalls that the adjustment of the diets to an equal nitrogen content resulted in concentrations of proline and glutamic acid which in molar equivalents were 4 times that of arginine. Since interconversions of these amino acids *in vivo* doubtless occur in molecular ratios, the efficiency of proline and glutamic acid as growth stimulants is seen to be even less than would appear to be the case from the relative increments in gains.

In contrast to the effects of proline and glutamic acid, hydroxyproline not only fails to accelerate growth but, when included in the diet to the extent of 3.41 per cent, actually exerts an inhibitory action. Thus, the mean gain of nine animals upon this level of hydroxyproline was  $26.2 \pm 1.7$  gm. as compared to a mean gain of  $49.1 \pm 0.85$  gm. for the negative controls. In view of the pronounced effect of hydroxyproline at this level, tests were carried out with a diet containing a smaller percentage of the amino acid. For this purpose, twenty male rats from three litters were divided into two groups each containing the same number of animals from each litter. One group received the basal diet supplemented with 1.0 per cent of hydroxyproline, and the other a diet containing an identical amount of nitrogen without hydroxyproline. The results are summarized in Table IV, Series III, and demonstrate that the rats which received hydroxyproline gained 5.1 gm. less than the negative controls. Whether this is a significant difference or not is a matter of judgment. A statistical analysis of the data shows that the ratio of the mean difference to the probable error of the difference is 3.8. This implies that the odds against the difference being due to chance alone are about 95 to 1. In any event, the tendency is in the direction of slower, rather than more rapid, growth when the ration contains as much as 1.0 per cent of hydroxyproline. Furthermore, it appears certain that this amino acid is incapable of improving the quality of a ration which is devoid of arginine, proline, and glutamic acid.

With respect to the arginine data in Table IV, attention is called to the fact that the mean growth increment is not so large as that observed in the third series of tests conducted under different experimental conditions by Borman *et al.* (1). The purpose of the latter investigation was to establish the growth effect of arginine when added to a diet which already contained

0.2 per cent of proline, 0.1 per cent of hydroxyproline, and 2.0 per cent of glutamic acid. Of the animals employed in Series III of that study only nine were males. Of these, four were deprived of arginine, and showed a mean gain in 28 days of 42.0 gm. Five litter mates received this amino acid, and manifested a mean gain of 82.0 gm. Thus, the increment in weight due to arginine, under the conditions specified, amounted to 40 gm. It should be emphasized that a larger number of animals was not used in this particular series, inasmuch as tests upon more than 300 rats had already demonstrated that arginine possesses a growth-stimulatory action. Thus, Series III was merely a confirmatory test under improved dietary conditions.

No experiments analogous to those of Borman *et al.* were conducted in the present investigation. As was pointed out above, the mean weight increment due to arginine when this amino acid was added to a diet *devoid* of proline, hydroxyproline, and glutamic acid amounted to 29.1 gm. Perhaps little significance should be attached to this divergence in view of the small number of animals used in the earlier experiment, and the differences in composition of the diets. In the light of the results recorded in Table IV of the present paper one would anticipate that the presence in the diet of proline and glutamic acid, even in the amounts used by Borman *et al.*, would have induced better growth in the negative controls than was actually observed. On the other hand, as will be demonstrated below, the mean gain of the animals which received all four amino acids also is not quite so great as is now being obtained. Therefore, the *increment of 40 gm.* due to arginine is probably not far from the correct value.

Having seen that arginine, proline, and glutamic acid, in contrast to hydroxyproline, are capable individually of stimulating the growth of animals, although arginine to a greater extent than either of the other two, it became necessary to compare the growth effects of arginine alone with that of all four amino acids administered simultaneously. For this purpose, two series of experiments were carried out. In one (Series IV) the nitrogen of the two diets was kept at the same level. This involved the use in one diet of a relatively high proportion of arginine (1.1 per cent, equivalent to 1.33 per cent of the monohydrochloride), comparable to that employed in Series I. In order to exclude the possibility that this level might have exerted a slight toxic effect, such as that observed with hydroxyproline, the test was repeated (Series V) with a lower percentage of the amino acid (0.4 per cent, equivalent to 0.5 per cent of the monohydrochloride). Under these conditions, the nitrogen content of the two diets obviously was not identical.

The results of both series of tests are summarized in Table V. No significant difference was observed in the growth effects of arginine at the

two levels, nor in the gains of the two groups of animals which received all four amino acids. On the other hand, both series of tests demonstrate that arginine alone is not so effective as is a mixture of the four amino acids. In Series IV, the ratio of the mean difference in growth of the two groups to the probable error of the difference is 3.9. This implies that the odds against the occurrence of a ratio as great or greater than this, due to

TABLE V

*Comparative Growth Effects of (1) Arginine and (2) Arginine Plus Related Amino Acids\**

The experiments covered 28 days each.

Series No.	Supplements	Amount in diet	No. of animals	Mean gain in weight and probable error of mean
		<i>per cent</i>		<i>gm.</i>
IV	Arginine	1.1†	22	80.0 ± 1.31
	"	0.4†		
	Proline	0.2	27	88.6 ± 1.79
	Hydroxyproline	0.1		
V	Glutamic acid	2.0	30	77.3 ± 1.18
	Arginine	0.4†		
	"	0.4†	27	90.6 ± 1.75
	Proline	0.2		
VI	Hydroxyproline	0.1	14	91.2 ± 1.66
	Glutamic acid	2.0		
	Arginine	0.6†	15	96.9 ± 1.48
	"	0.6†		
	Proline	0.6	15	91.0 ± 1.77
	Arginine	0.6†		
	Hydroxyproline	0.3	15	102.0 ± 2.04
	Arginine	0.6†		
	Glutamic acid	2.0	16	105.8 ± 2.19
	Arginine	0.6†		
	Proline	0.6		
	Hydroxyproline	0.3		
	Glutamic acid	2.0		

\* Each supplementary amino acid possessed the L configuration.

† Administered as the monohydrochloride (1.33, 0.5, and 0.73 per cent of the diet respectively) with an equivalent amount of sodium bicarbonate.

chance alone, are 116 to 1. Most investigators would regard this as statistically significant. In Series V, the corresponding ratio is 6.3, which indicates a highly significant difference. Incidentally, it is of interest that a comparison of the mean gain of the animals which were deprived of all four amino acids (Series I, Table IV) with the mean gain of the animals which received these compounds (Series IV and V, Table V) reveals differences

of 39.5 and 41.5 gm., respectively. While these increments in gain were obviously not strictly comparable to those observed by Borman *et al.* and referred to above, they are of the same order, and suggest that the quantities of proline and glutamic acid used in the earlier investigation may not be very effective growth stimulants in the absence of arginine from the diet.

The beneficial effects of proline and glutamic acid, when included in a diet containing arginine, were unexpected in view of our previous experience with these amino acids. Moderate differences in growth such as these could not have been detected under dietary conditions employed formerly in this and other laboratories. As explained elsewhere (1), a systematic effort has been made in recent years to improve the nutritive quality of our amino acid diets. As a result, we are now able to obtain much more satisfactory growth, when all required amino acids are present in the food, than was possible originally. Likewise, moderate deficiencies are now revealed much more clearly than was previously the case. This is illustrated by the larger increment in weight induced by arginine in animals receiving the revised, as contrasted with the older, basal ration (*cf.* Series I and III (1)).

In consequence of the greater gains of the animals which received all four compounds, additional tests were undertaken to establish whether the improvement was attributable to one or was characteristic of each of the amino acids in question. For this purpose, 75 male rats from thirteen litters were divided as equitably as possible into five groups of approximately equal number. One group received a diet containing 0.6 per cent of arginine (equivalent to 0.73 per cent of the monohydrochloride) as the sole amino acid supplement. This level of arginine is a liberal but not an excessive one. Three groups of animals received rations containing the same percentage of arginine in addition to either 0.6 per cent of proline, 0.3 per cent of hydroxyproline, or 2.0 per cent of glutamic acid. The fifth group received the four amino acids, each at the percentage used in the other diets of this experiment. The results are summarized as Series VI in Table V.

Unfortunately, the weight gains of the rats in each group were rather variable as indicated by the relatively large probable errors of the means. However, the data reveal several facts of considerable interest. In the first place, no difference is evident in the growth of the animals which received arginine alone ( $91.2 \pm 1.66$  gm.) and arginine plus hydroxyproline ( $91.0 \pm 1.77$  gm.). Obviously, the presence of this amino acid does not improve the quality of a diet containing arginine, nor does it exert detectable deleterious effects in the quantity here employed. The animals which received arginine plus proline showed a mean gain of  $96.9 \pm 1.48$  gm. This is 5.7 gm. in excess of the mean gain of the controls which received

arginine alone. This divergence is not statistically significant, as is indicated by the fact that the ratio of the mean difference to the probable error of the difference is only 2.6. On the other hand, the animals which received arginine plus glutamic acid, and arginine plus the prolines and glutamic acid, showed increments in weight over the arginine controls of 10.8 and 14.6 gm., respectively. Both of these increments are statistically significant, inasmuch as the ratio of the mean difference to the probable error of the difference in the two groups is 4.1 and 5.3, respectively. The divergence in the mean gain of the animals which received arginine plus glutamic acid, as compared with arginine plus the prolines and glutamic acid, is 3.8 gm. This is not statistically significant. Apparently, therefore, the better growth brought about by the combination of four amino acids is due largely, if not entirely, to the presence of glutamic acid. A more positive interpretation would be hazardous in the extreme, since the differences in such experiments are small and the "biological" variations are relatively large. Furthermore, despite the results of the statistical analyses, one cannot escape the impression that the *tendency* is toward progressively better growth when proline, glutamic acid, and both of these amino acids are added to a diet containing arginine. This is what one would anticipate if the data carry the implication which tentatively we believe they do.

Before undertaking to interpret the findings in the investigation as a whole, attention must be directed parenthetically to one other observation of an unexpected nature. Throughout the experiments summarized in Series I to V inclusive, comparable tests yielded remarkably uniform results. Thus, three groups of animals which received the basal diet without an amino acid supplement showed mean gains of 49.1, 48.0, and 48.2 gm., respectively. Three groups upon rations containing arginine as the sole supplement, at different but adequate levels, manifested mean gains of 78.2, 80.0, and 77.3 gm., respectively. Finally, two groups of rats which received all four amino acids exhibited mean gains of 88.6 and 90.6 gm., respectively. With Series VI, however, this uniformity ceased. In the latter series, the gains were invariably larger than those attained by the subjects in corresponding tests in the preceding experiments. Thus, upon the diet carrying arginine alone the mean gain was 91.2 gm., and upon the ration containing all four amino acids the mean increase in weight was 105.8 gm. Obviously, this improvement in growth calls for an explanation, although it does not alter the validity of the comparisons already made between the various groups within Series VI.

In the conduct of the tests in Series VI one modification of the basal diet was instituted, namely the replacement of DL-tryptophan by an equal weight of L-tryptophan. This would seem to be an inconsequential altera-

tion, inasmuch as the optical isomers of this amino acid are said to be equally effective in the rat for growth purposes (13, 14). Despite our skepticism that this substitution might have been responsible for the increased gains in Series VI, experiments were undertaken to compare the effects of L- and DL-tryptophan under otherwise identical conditions. These tests are still in progress, but to date no level of the racemic compound has permitted as satisfactory growth as the same level of the L isomer. The explanation of this apparent superiority of L-tryptophan must await the completion of additional tests.

#### DISCUSSION

The data in Table IV demonstrate clearly and unmistakably that in the rat proline and glutamic acid, like arginine, exert growth-stimulatory effects when added separately to a basal diet which is devoid of these amino acids. Thus, the three compounds possess the same activity qualitatively, although from the quantitative point of view arginine is much superior to either of the other two. Ordinarily, a diet from which two or more essential amino acids have been removed *completely* is not improved for growth purposes by the addition of one of the missing components. This was brought home forcibly to us in the course of the isolation and identification of threonine. The basal ration then used in assaying the protein fractions was, for unavoidable reasons, deficient in isoleucine. This condition could not be demonstrated, however, until threonine became available, since, in the absence of the latter, the addition of isoleucine failed to improve the quality of the food (*cf.* Womack and Rose (15)). Such a relationship is to be expected in multiple deficiencies of a complete nature, since the absence of a second essential still renders the ration unsatisfactory for tissue synthesis.

The situation in the present study is different in that the amino acids in question can be manufactured by the rat, at least to a limited extent, and consequently, though excluded from the food, are still not entirely wanting to the cells. Nevertheless, the ability of three amino acids, when administered singly, to accelerate the growth of animals which have been deprived of them simultaneously has no counterpart. This unique situation is of paramount importance in the interpretation of the findings. The only logical explanation, it seems to us, is one which assumes that arginine, proline, and glutamic acid are mutually interconvertible, but at different rates, as exemplified by their varying effects. Evidence that certain of these interconversions actually occur has already been cited. The present study points strongly to the probability that glutamic acid is converted into arginine or proline. This has not been observed *in vivo* previously. On the other hand, hydroxyproline either is not transformed



into the other members of the group, or the transformation takes place too slowly to be detected by the growth method. This difference in the behavior of hydroxyproline is not very surprising, inasmuch as several investigators have called attention to the fact that the rates of oxidation or the pathways of metabolism of proline and hydroxyproline are not identical (16, 4, 5, 17).

The data in Table V indicate that even arginine, the most potent of the four amino acids under investigation, is incapable of effecting as satisfactory growth as when all are included in the food. Under the latter condition, much of the supplementary activity is attributable to the glutamic acid though proline may be helpful to a very limited extent. These observations may be interpreted in at least two ways. The first alternative is to regard glutamic acid as an essential amino acid under the definition postulated in this laboratory to the effect that such a compound is "one which cannot be synthesized by the animal organism, out of materials *ordinarily available* to the cells, . . . at a speed commensurate with the demands for *normal growth*" (1). Perhaps the classification of glutamic acid in this way would be the more consistent course of action. However, as pointed out above, glutamic acid is much less effective than arginine when administered alone. Furthermore, even when the diet contains 2.0 per cent of glutamic acid, together with small amounts of proline and hydroxyproline, the addition of arginine greatly accelerates growth (1). All tests appear to point to arginine as the critical member of the group. In view of this fact, we are *tentatively* adhering to the other obvious alternative; namely, the postulate that the three amino acids are mutually interconvertible, and that differences in their activity when administered alone or in combination are referable to the rates at which they are transformed into each other. We have emphasized on several occasions in other connections that the classification of an amino acid like arginine or glutamic acid as dispensable or indispensable is purely a matter of definition. The important point is the demonstration that, though beneficial in the diet, they are very different from amino acids like tryptophan, leucine, phenylalanine, etc., which *apparently* cannot be synthesized at all in the organism of the rat.

Finally, it is worthy of note that reactions of interconversion are not the only means whereby arginine, proline, and glutamic acid can be manufactured *in vivo*. This is shown clearly by the fact that moderate growth occurs when all three compounds, in addition to hydroxyproline, are excluded from the food.

#### SUMMARY

Experiments have been conducted to determine the dietary significance of proline, hydroxyproline, and glutamic acid. For this purpose, tests

have been carried out upon more than 300 young rats. The data demonstrate that the addition of proline or glutamic acid to a diet which is devoid of the three amino acids in question and of arginine leads to a statistically significant increase in the mean gain of the subjects. Neither proline nor glutamic acid is nearly as effective as arginine. Under like circumstances, a moderate amount (1.0 per cent of the diet) of hydroxyproline exerts no beneficial effect, and a larger proportion (3.41 per cent) inhibits growth.

The above findings are interpreted as indicating that arginine, proline, and glutamic acid are mutually interconvertible in the organism of the rat, but at different rates as exemplified by their different influence upon growth. The glutamic acid data provide indirect evidence, for the first time, that this amino acid may be transformed *in vivo* into proline or arginine. Hydroxyproline either is not converted into the other members of the group, or the reaction proceeds too slowly to be detected by the growth method.

Comparisons of the effects of arginine alone, and of arginine, proline, hydroxyproline, and glutamic acid administered simultaneously, demonstrate that the rate of gain is moderately improved when all four amino acids are present in the food. The superiority of the latter ration is due largely, if not entirely, to the inclusion of glutamic acid. A ration containing proline and arginine fails to induce a statistically significant improvement in growth over one containing arginine alone.

Interconversions of arginine, proline, and glutamic acids are not the only means whereby these amino acids can be manufactured *in vivo*. This is made evident by the fact that moderate growth occurs when all three compounds, in addition to hydroxyproline, are excluded from the food.

Hydroxyproline has been shown to be a dispensable amino acid. Proline also belongs to the dispensable group but, in the growing rat, can replace in part the arginine requirement. The status of glutamic acid is less certain. *Tentatively* it is regarded as non-essential, but its final classification must await the results of additional experiments.

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BIOTIN, CHOLINE, INOSITOL, *p*-AMINOBENZOIC ACID,  
AND VITAMIN B<sub>6</sub> IN TRANSPLANTABLE MOUSE  
CARCINOMAS AND IN MOUSE BLOOD\*

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The levels of biotin, choline, inositol, *p*-aminobenzoic acid, and of the vitamin B<sub>6</sub> complex in mouse epidermis treated with the potent carcinogen, methylcholanthrene, have been determined by Tatum *et al.* (1) as part of a study of the mechanism of the production of epidermal cancers in mice. Except for one value for choline (2), there are no reports in the literature on the levels of B vitamins in methylcholanthrene-produced skin carcinomas. The amounts of biotin, choline, inositol, *p*-aminobenzoic acid, and of the vitamin B<sub>6</sub> complex in a transplantable squamous cell carcinoma have now been determined. Since it was not possible to obtain tumor samples free of blood, it was necessary also to assay mouse blood for these vitamins. This paper summarizes the results of these determinations.

EXPERIMENTAL

*Tumors*—The tumor type analyzed was a squamous cell carcinoma descended from a transplantable carcinoma which Cooper, Firminger, and Reller (3) had originally produced in the epidermis of a Swiss mouse by the topical application of methylcholanthrene. There are certain definite advantages in analyzing transplanted tumors rather than carcinomas individually produced by methylcholanthrene. As has been mentioned by Suntzeff and Carruthers (4), the transplanted tumors have less necrosis and keratinization and show more uniformity in the type of malignant cells present than do tumors individually produced upon the skin. Transplanted tumor tissue is less likely to contain connective tissue mixed with the tumor tissue. Finally, the transplanted tumors grow much faster than do the tumors arising on the skin.

The tumors were grown subcutaneously in 6 week-old Swiss mice of both sexes. Suntzeff and Carruthers (4) found that the rapidly growing trans-

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plants began to show some necrosis when less than 10 mm. in diameter, and therefore analyzed tumors 5 to 8 mm. in diameter. Except as indicated in Table II, we also have sacrificed the animals when the tumors measured from 5 to 8 mm. in diameter, about 12 days after implantation. Even tumors as small as this sometimes showed central necrosis. Tumors were collected from five different generations of transplants,<sup>1</sup> the most recent being the thirty-eighth generation. The microscopic appearance of the different transplanted tumors was quite similar, showing solid sheets of malignant epithelial cells with relatively slight keratin formation. In preparing the tumors for analysis, the connective tissue capsule was removed, the tumor blotted with a good grade of filter paper to remove adherent blood, any necrotic tissue scraped away, and the tumor cut into small pieces. Pooled samples were dried and stored *in vacuo* over phosphorus pentoxide, as previously described (1). Tumors from about twenty-five mice were required to give a dried sample weighing approximately 0.5 gm.

The preparation of the tumor tissue extracts for microbiological assay and the methods of assay of biotin,<sup>2</sup> choline, inositol, *p*-aminobenzoic acid, and the vitamin B<sub>6</sub> complex with normal and mutant strains of *Neurospora* were the same as were previously used for the corresponding analyses of mouse epidermis during methylcholanthrene carcinogenesis (1). These five members of the vitamin B complex make a convenient group for investigation, since the same method of tissue hydrolysis is adequate for each vitamin, and since each substance can be satisfactorily determined with a specific strain of *Neurospora* (1). In order to facilitate the liberation of the vitamins from the tissue by the sulfuric acid hydrolysis, the dried tumor samples were finely ground in a small mullite mortar and then redried before being used.

*Blood*—Tumors, at least when still comparatively small, are usually well supplied with blood vessels. Although some blood could be removed from the mouse tumors by blotting, the minced tumor tissue had a slight but definite pink color due to the presence of blood. It was therefore necessary to determine whether any of the vitamin B factors under examination were present in mouse blood in amounts sufficiently high to affect the tumor determinations. In the previous assays of epidermis it had not been necessary to consider the vitamin levels of the blood, since epidermis is avascular.

Healthy female Swiss mice, about 8 weeks old, which had been fed Rockland mouse pellets and water *ad libitum*, were anesthetized with ether,

<sup>1</sup> The tumors used for transplanting were obtained through the courtesy of Dr. V. Sontzeff.

<sup>2</sup> The biotin used in this work was kindly supplied by Merck and Company, Inc., Rahway, New Jersey.

and venous blood was drawn from the right ventricle of the heart or from the inferior vena cava. In all cases, the blood was withdrawn at the same time of day, about 2 p.m. The blood was transferred from the syringe to a weighing bottle containing a weighed amount of sodium citrate which had been oven-dried in the bottle. On an average, 0.4 gm. of blood was obtained per mouse. The removal of this amount of blood resulted in the death of the animals. Since the blood was being studied in relationship to the tumors, the blood samples were dried, ground, extracted, and assayed in the same manner as the tumor samples.

The values obtained for the content of biotin, choline, inositol, *p*-aminobenzoic acid, and vitamin B<sub>6</sub> in mouse blood are given in Table I, calculated in micrograms per gm. of dry weight, in order to facilitate comparison with the tumor results. Both tissues had a water content of 80.5 per cent as

TABLE I  
*Content of Five Vitamin B Factors in Mouse Blood*

The values are given for pooled samples of blood and are expressed in micrograms per gm. of dried blood, with the averages in parentheses.

Vitamin B factor	Sample I	Sample II	Sample III	Sample IV	Sample V	Over-all average
Biotin		0.030	0.048 0.053 (0.050)	0.144 0.157 (0.150)	0.021 0.032 (0.026)	0.064
Choline	1165	894	326 546 339 (404)	623 478 740 (614)	1658	947
Inositol	330	218	326	395	410	336
<i>p</i> -Aminobenzoic acid	1.54	1.54	1.46	1.40		1.49
B <sub>6</sub> complex			2.10	2.36	2.01	2.16

determined by drying for 60 hours *in vacuo* over phosphorus pentoxide.

No figures on the levels of any of these five vitamins in mouse blood were found in the literature. Levels of inositol and vitamin B<sub>6</sub> have not previously been reported for the blood of any animal. As is shown in Table I, some of the blood samples, each of which consisted of the blood from four mice, differed considerably in biotin content. The average biotin value was 0.064  $\gamma$  per gm. of dry weight, or 0.0125  $\gamma$  per cc. Burk *et al.* (5) reported values of 0.015 to 0.035  $\gamma$  per cc. in the blood of rats being fed *p*-dimethylaminoazobenzene. Our results indicate also considerable variations among mice in the choline content of blood. This probably reflects variations in lecithin. The average choline value was 947  $\gamma$  per gm. of dry weight, or 185  $\gamma$  per cc. This is about the same as Luecke and Pearson reported for the plasma of horses and cattle (6) and of dogs (7).

The blood inositol level varied less than did the biotin and choline levels. The average value for inositol was 336  $\gamma$  per gm. of dry weight, or 66  $\gamma$  per cc. *p*-Aminobenzoic acid and vitamin B<sub>6</sub> showed almost no variations among the different groups of mice. *p*-Aminobenzoic acid averaged 1.49  $\gamma$  per gm. of dry weight, or 0.29  $\gamma$  per cc. Pennington found 0.03  $\gamma$  per cc. of *p*-aminobenzoic acid in human blood (8). The vitamin B<sub>6</sub> assays gave an average value for mouse blood of 2.16  $\gamma$  per gm. of dry weight, or 0.42  $\gamma$  per cc.

The values obtained for the five vitamin B factors in the transplantable mouse tumor are summarized in Table II. As in the studies on epidermis (1), dry weight of tissue was used as the basis of reference. It was found

TABLE II

*Content of Five Vitamin B Factors in Transplanted Mouse Carcinoma*

The values are given for five pooled samples of tumors, and are expressed in micrograms per gm. of dried tumor, with the averages in parentheses.

Vitamin B factor	Sample I	Sample II	Sample III	Sample IV	Sample V*	Over-all average
Biotin	0.162	0.140	0.158	0.133	0.154	0.149
Choline	5550 6525 (6038)	6450	6100 5900 (6000)	6602	6108	6240
Inositol	894 952 969 (938)	1164 995 (1080)	1255 1310 (1282)	1043	1427	1154
<i>p</i> -Aminobenzoic acid	3.64	2.95	3.80	2.28	2.78	3.09
B <sub>6</sub> complex	3.10 3.85 (3.48)	4.64 4.29 (4.46)	2.99 4.10 (3.55)	5.54 3.56 (4.55)	4.47	4.10

\* Tumors harvested at 21 days.

unnecessary to correct the tumor values for blood for the following reasons: Surtzoff and Carruthers (4), by determining the iron content of mouse blood and of transplanted carcinomas, showed that, even if all of the iron in the tumors were present as hemoglobin, the blood content of the tumors could not exceed 5 per cent. It is probable that much of the iron in the tumors is not hemoglobin iron, since the completely avascular hyperplastic epidermis which gives rise to tumors contains about the same amount of iron (9) as the tumors. Our results show approximately 50 per cent as much *p*-aminobenzoic acid and vitamin B<sub>6</sub> in blood as in the tumors, while the other vitamins were found in the blood at lower relative levels. When the amounts of the vitamins in the tumors were corrected for blood vitamin

content, assuming 5 per cent blood in the tumors, it was found that the resultant changes in the tumor values were negligible. For each of the vitamin B factors, the change in the tumor value was less than the spread between replicate assays of the same tumor sample.

For the tumor samples, the levels of the individual vitamin B factors varied less from one sample to another than was the case with the blood samples. This may have been due to the fact that, whereas four mice were represented in each blood sample, twenty-two to forty-one mice were represented in each tumor sample, except tumor Sample V (Table II). This latter sample consisted of only seven tumors, which were collected 21 days after implantation, and were therefore considerably larger, as well as somewhat more necrotic, than the 12 day carcinomas comprising the other samples. Assays for choline, inositol, and vitamin B<sub>6</sub> were repeated on some of the tumor hydrolysates because of a spread in the values for these vitamins (particularly B<sub>6</sub>) among the different groups of tumors. The results of the different assay runs agreed fairly well (Table II).

The average biotin content of the transplantable mouse carcinoma was 0.149  $\gamma$  per gm. of dry weight, and the *p*-aminobenzoic acid values averaged 3.09  $\gamma$  per gm. of dry weight. No figures were found in the literature which can validly be compared with these values. The average of 6240  $\gamma$  of choline per gm. of dry weight found for the mouse carcinoma is about the same as that reported by Haven and Levy (10) for transplanted rat carcinosarcoma 256. The average value found for inositol in the mouse tumor, 1154  $\gamma$  per gm. of dry weight, is higher than the values reported by Pollack, Taylor, and Williams (11) for cancers of the skin and breast in mice, and the pyridoxine levels reported by these authors were somewhat lower than our findings for the whole vitamin B<sub>6</sub> complex. The average vitamin B<sub>6</sub> content of our transplantable mouse carcinoma was 4.10  $\gamma$  per gm. of dry weight.

Table III compares the levels of the vitamin B factors in the transplanted carcinomas with their levels in normal and treated epidermis. By using the technique of Baumberger, Suntzeff, and Cowdry for heat separation of the two layers of the skin, epidermis uncontaminated by dermis can easily be obtained (12) and serves as an excellent control tissue for use in chemical studies of epidermal carcinogenesis (9). Similarly, epidermis rendered hyperplastic by the topical application of 0.6 per cent methylcholanthrene in benzene is obtained entirely free from dermis. Therefore, values of vitamin B factors in the transplanted tumors, which were descended from an epidermal carcinoma produced by application of methylcholanthrene and which had continued to be squamous cell carcinomas, can validly be compared with the values found for normal epidermis, for



epidermis painted with methylcholanthrene in benzene, and for epidermis painted with benzene alone (1).

Of the five vitamins studied, all except biotin increased in the tumors as compared both with the normal and the treated epidermis. Choline showed the greatest change, the tumor value being 2.5 times greater than in normal epidermis. This compound increased only slightly in the methylcholanthrene-treated epidermis, the rise perhaps being due to the benzene rather than to the carcinogen, since the elevation was more marked in the benzene-treated epidermis. Jacobi and Baumann (2) found less choline in a mouse skin cancer than in several normal visceral organs, but did not study skin or isolated epidermis. The level of inositol, which had been very little affected by treatment of the epidermis over a 2 month period, rose in the tumors to 219 per cent of that in normal epidermis. *p*-Amino-

TABLE III

*Summary of Content of Five Vitamin B Factors in Mouse Tissues during Methylcholanthrene Carcinogenesis*

The values given are expressed in micrograms per gm. of dried tissue, and as per cent of the values for normal epidermis.

B factor	Normal epidermis	Benzene-treated epidermis		Methylcholanthrene-treated epidermis		Transplanted carcinomas	
	$\gamma$ per gm.	$\gamma$ per gm.	per cent	$\gamma$ per gm.	per cent	$\gamma$ per gm.	per cent
Biotin	0.196	0.194	99	0.125	64	0.149	76
Choline	2471	2802	113	2651	107	6240	252
Inositol	526	568	108	547	104	1154	219
<i>p</i> -Aminobenzoic acid	2.40	2.30	96	2.40	100	3.09	129
B <sub>6</sub> complex	2.45	2.88	118	3.05	124	4.10	167

benzoic acid behaved somewhat similarly in that the level of this factor showed no change with methylcholanthrene or with benzene alone, but was somewhat increased in the carcinomas, reaching 129 per cent of normal. The value for vitamin B<sub>6</sub> increased from 118 per cent of normal in the benzene-treated epidermis, to 124 per cent in the methylcholanthrene-treated epidermis, and to 167 per cent in the tumors.

Of the five vitamin B factors, biotin alone decreased in the methylcholanthrene-painted epidermis. The biotin value was also the only one which in the tumors fell below that for normal epidermis. However, its level in the carcinomas, 76 per cent of normal, was slightly higher than in the hyperplastic epidermis, where it was 64 per cent of normal. West and Woglom (13) and Kidd, Winzler, and Burk (14) determined biotin in rabbit skin tumors, but both these groups of investigators emphasized the difficulties of obtaining good control tissue for analysis. Both groups

found biotin higher in the tumors than in the skin, but did not assay isolated epidermis.

#### DISCUSSION

It is perhaps significant that the two factors which showed the greatest rise in concentration in the carcinoma tissue compared with their concentrations in normal epidermis are known to be concerned in lipide metabolism and to occur as constituents of animal lipides. Choline rose to a level of 252 per cent of that in normal epidermis, and inositol increased to 219 per cent. The increase in choline, as a constituent of lecithin, may well be related to the increased phospholipide content of mouse epidermal tumors, both induced and transplanted, found in other experiments by one of us.<sup>3</sup> The increase in inositol may also be correlated with phospholipide if inositol is present in tumors in a combined form such as the inositol-containing phosphatides found by Folch (15) in brain "cephalin." These considerations might suggest the possibility of modifying tumor growth by controlling the availability of choline and inositol. The available experimental results bearing on this question are difficult to interpret. Jacobi and Baumann (2) failed to detect any effect of the dietary choline level on the survival time of mice bearing epithelial tumors produced by methylcholanthrene. Laszlo and Leuchtenberger (16) claimed that inositol given intravenously markedly decreased the size of tumors in mice in a 48 hour period, but found that oral or subcutaneous administration of inositol had no effect.

The changes in the levels of the other three vitamins studied may be interpreted as accompanying alterations in nitrogen metabolism in the tumor, since each vitamin has been implicated more or less directly with some phase of metabolism of nitrogen compounds. The rôle of vitamin B<sub>6</sub> in amino acid metabolism and synthesis has been well established (17-19). The suggestion from the work of Winzler, Burk, and du Vigneaud (20) that biotin is concerned with nitrogen assimilation has been strengthened by the finding of Stokes, Larsen, and Gunness (21) that this vitamin plays a rôle in aspartic acid synthesis in lactic acid bacteria. In addition to the presence of *p*-aminobenzoic acid in the folic acid molecule (22), this substance has been linked with the synthesis of methionine and certain purines in bacteria (23-25).

One is perhaps justified in viewing the change from normal epidermis to carcinoma as a temporal sequence of changes in the biochemistry and metabolism of the cells involved. From this aspect, a change which is evident in early carcinogenesis with methylcholanthrene may be more directly related to the process of carcinogenesis than a change detectable

<sup>3</sup> Wicks, L. F., unpublished findings.

only at a later stage in the developed tumor. The data summarized in this paper indicate, in line with this interpretation, that biotin is of primary significance in methylcholanthrene carcinogenesis. This may also be true for vitamin B<sub>6</sub>, since the validity of the increase previously found for hyperplastic epidermis (1) is strengthened by the still higher value found for the tumor. Perhaps the other three vitamin B factors, choline, inositol, and *p*-aminobenzoic acid, are less directly concerned with carcinogenesis than with growth and metabolism of the tumor itself, since none of these factors changed significantly in epidermis during methylcholanthrene-induced hyperplasia.

Information is now available on the changes in lipides (26), in ascorbic acid and some of the metals (27), and in five members of the vitamin B complex during epidermal carcinogenesis. However, more information on the biochemistry of carcinogenesis, as well as on the metabolic functions of all of these tissue constituents, will be required for a critical evaluation of the significance of the changes so far observed.

#### SUMMARY

The levels of biotin, choline, inositol, *p*-aminobenzoic acid, and vitamin B<sub>6</sub> have been determined in a transplanted epidermal carcinoma in mice and in mouse blood. *Neurospora* mutant strains were used for the bioassays.

The levels of these vitamin B factors in mouse blood are lower than in the epidermal carcinoma.

Choline and inositol, which had shown little change in methylcholanthrene-treated epidermis, increased most in the tumors, choline reaching 252 per cent of the value for normal epidermis and inositol 219 per cent of the normal.

The concentration of biotin in the tumors was somewhat below the level for normal epidermis, but was not as low as in the hyperplastic epidermis.

The vitamin B<sub>6</sub> complex, which had risen to 124 per cent in the hyperplastic epidermis, was elevated to 167 per cent in the carcinoma.

*p*-Aminobenzoic acid, which had remained at the normal level in the treated epidermis, increased to 129 per cent in the tumors.

The possible significance of these results is discussed in relation to methylcholanthrene-induced epidermal carcinogenesis.

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## STEROID LACTONES

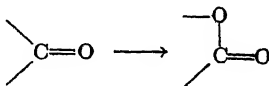
### I. THE OXIDATION OF ESTRONE ACETATE TO THE LACTONE ACETATE OF ESTROLIC ACID\*

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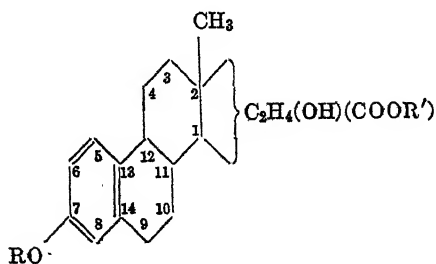
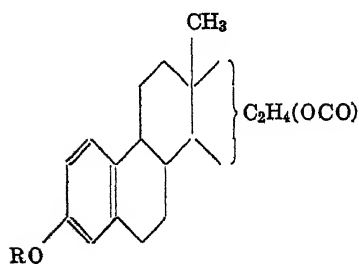
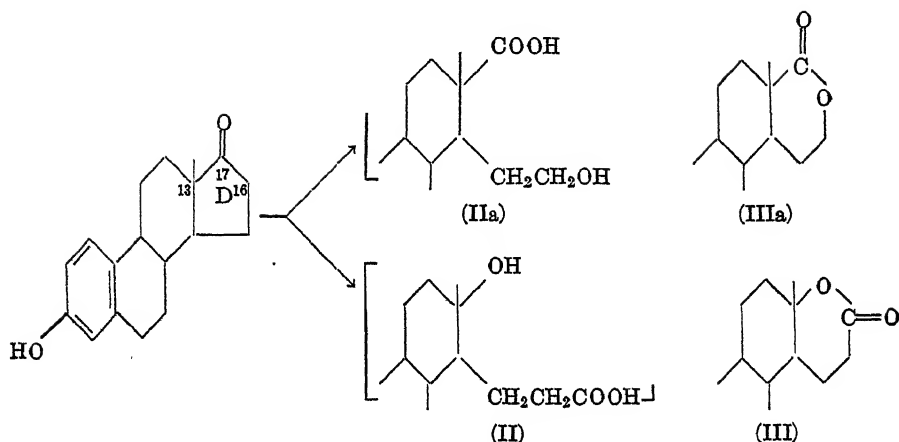
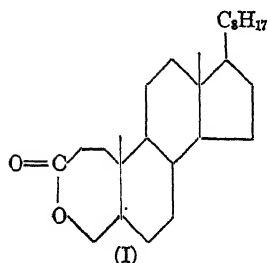
The oxidation of a steroid ring ketone to a lactone according to the scheme



was first reported by Gardner and Godden (1), who obtained two lactones from the ammonium persulfate oxidation of coprostan-3-one. The production of one of these was later confirmed by Burckhardt and Reichstein (2), who obtained the lactone (I) by perbenzoic acid oxidation of coprostanone and reported the preparation of similar lactones from cholestan-3-one, methyl 3-keto-12( $\alpha$ )-acetoxycholanate, methyl 3-keto-11-cholenate, and methyl 3-ketocholanate. These investigators reported further that under the conditions employed for the 3-keto compounds neither methyl 3( $\alpha$ )-acetoxo-12-ketocholanate nor allopregnan-3( $\alpha$ )-ol-20-one acetate formed lactones. Recently, other lactones from 3-keto steroids have been prepared by Ruzicka, Prelog, and Meister (3) in connection with a study of the relationship between the structure and odor of certain androstane and etiocholane derivatives.

Westerfeld (4) has reported the oxidation of the 17-keto steroid, estrone, with alkaline hydrogen peroxide to form in low yield a sparingly soluble  $C_{18}$ -lactone melting at  $335^\circ$ , which was characterized by the formation of an acetate melting at  $143.5$ – $145^\circ$  and a methyl ether melting at  $166$ – $168^\circ$ . He formulated the reaction as involving the cleavage of Ring D, either between carbon atoms 16 and 17 or, more probably, 13 and 17, followed by the addition of the elements of hydrogen peroxide to give an intermediate dihydroxy acid (II), which lactonized on acidification of the reaction mixture to form III. Westerfeld, subsequently confirmed by Smith (5), established the estrogenic potency of the hydroxy lactone (III)

\* The work described in this paper was supported by a grant from G. D. Searle and Company.



IIIb. R = H—

IV. " = CH<sub>3</sub>CO—

V. " = CH<sub>3</sub>CH<sub>2</sub>CO—

VI. " = C<sub>6</sub>H<sub>5</sub>CO—

VII. " = CH<sub>3</sub>—

VIII. R = H—, R' = H—

IX. " = H—, " = Na—

X. " = CH<sub>3</sub>—, R' = H—

XI. " = H—, R' = CH<sub>3</sub>—

XII. " = CH<sub>3</sub>—, R' = CH<sub>3</sub>—

(actually an alkaline solution of the corresponding dihydroxy acid or "open form") as being about one-fifteenth that of estrone. The latter investigator reported further that this material stimulated the secretion of

gonadotropic and adrenocorticotropic hormones by the pituitaries of mature male rats to a much greater degree than could be accounted for alone by the estrogenic activity of the substance.

We have begun an investigation of the oxidation of a variety of keto steroids in order to prepare the lactones and their derivatives for physiological study. In this first report is described the oxidation of estrone acetate to form the lactone acetate (IV) of a dihydroxy acid (VIII) which for convenience has been given the name estrolic acid.

As a method for the preparation of estrolic acid derivatives, the oxidation procedure of Westerfeld was found to be unsuitable. Estrone acetate was therefore oxidized with a large excess of hydrogen peroxide in acetic acid solution. The crude crystalline lactone acetate, which was obtained in 57 to 63 per cent yield, was purified by alkaline hydrolysis, followed by carbonation of the aqueous alkaline solution to precipitate a small quantity of unaltered estrone. The purified hydroxy lactone (IIIb), which was obtained by subsequent acidification and heating, was then acetylated to produce pure IV; the corresponding propionate (V) and benzoate (VI) were also prepared.

The methyl ether (VII) of IIIb was prepared directly with methyl iodide and silver oxide or by dissolving (with saponification) the lactone in hot sodium hydroxide and treating the solution with dimethyl sulfate. From the alkaline saponification mixture of IIIb was also obtained the parent dihydroxy acid (VIII) by careful acidification with mineral acid in the cold, while the partial acidification of this alkaline solution with carbon dioxide afforded the crystalline sodium salt (IX) of the free phenol. The methyl ether (VII) was similarly saponified and the resulting chilled solution was acidified to produce the methyl ether acid (X). Treatment of the carboxylic acids VIII and X with ethereal diazomethane formed the methyl esters XI and XII, the first of which on treatment with benzoyl chloride in pyridine solution formed the benzoate of IIIb, instead of that of XI, together with a non-crystalline fraction. The latter, after alkaline hydrolysis, yielded a small crystalline fraction, m.p. 167–171°, which could not be successfully purified for analysis.

In an effort to characterize the third oxygen function of estrolic acid (VIII), several unsuccessful attempts were made to dehydrate its methyl ester; in all experiments the principal product was the hydroxy lactone (IIIb), with only traces, too small to manipulate, of other crystalline alteration products.

Pursuing this aim further, it seemed to us reasonable to suppose that the lactone itself, if it is of the  $\delta$ -substituted  $\delta$ -lactone type, might undergo Clemmensen reduction<sup>1</sup> to give a mixture of stereoisomeric anhydrodihydro-

<sup>1</sup> Unpublished observations. See Martin, in Adams, R., *Organic reactions*, New York, 1, 162 (1942).



estrollic acids. The reduction of the lactone acetate (IV) with amalgamated zinc and hydrochloric acid in acetic acid was found to proceed smoothly, with the production of a mixture of crystalline acids, the resolution of which is being undertaken and will be described later.

The formulation of estrollic acid (VIII) as a 1,2-disubstituted 2-methyl-7-hydroxy-1,2,3,4,9,10,11,12-octahydrophenanthrene rests largely on its preparation from estrone and on the functional derivatives described above. The arrangement of the substituents at C<sub>1</sub> and C<sub>2</sub> as in IIa seems improbable on the grounds that this structure resembles that of the polyhydrophenanthrene carboxylic acids of the marrianolic acid type, in the esters of which the tertiary carbomethoxyl has repeatedly<sup>2</sup> been observed to be more resistant to alkaline hydrolysis than the primary carbomethoxyl of an adjacent (C<sub>1</sub>) acetic or propionic ester group. The carbomethoxyl group in XI appears to be of the latter type, since it is easily hydrolyzed by warm, aqueous, methanolic potassium bicarbonate to form the hydroxy lactone (IIIb) in nearly quantitative yield.

It should be noted further than an acid of the partial structure IIa of the doisyolic acid type of compound would be expected to possess relatively high estrogenic activity. Actually, estrollic acid and its derivatives show less than 0.01 the activity<sup>3</sup> of estrone.

Among the derivatives (IIIb, IV, and VII) of estrollic acid reported here which parallel those of III prepared by Westerfeld, there may exist chemical similarities, but their identity is seriously open to question on physiological grounds. Westerfeld's lactone (III) in the Allen-Doisy assay is over 6 times more active than IIIb administered in the same manner and, more strikingly still, it possesses pituitary-stimulating action,<sup>4</sup> while estrollic acid and its derivatives actually inhibit<sup>5</sup> the secretion of gonadotropic hormone by the pituitary glands of immature female rats.

Work in this laboratory is continuing on the oxidation of a variety of keto steroids and on the elucidation of the structure and configuration of the products obtained.

#### EXPERIMENTAL<sup>5</sup>

*Oxidation of Estrone with Alkaline Hydrogen Peroxide*—Estrone (0.60 gm.) was oxidized at 30° in two experiments according to the method of

<sup>2</sup> Numerous examples are to be found in the publications of Bachmann and co-workers on the synthesis of sex hormones (see (6) for the most recent paper of this series), and those of Miescher *et al.* ((7) Paper XVI) on the estrogenic carboxylic acids.

<sup>3</sup> A full report of the physiological studies will be made in a separate paper.

<sup>4</sup> This has been observed (private communication from Dr. O. W. Smith) in immature female rats as well as in mature males.

<sup>5</sup> All melting points were determined in open capillaries with total immersion of

Westerfeld (4). The combined solid (0.36 gm.) obtained by acidification of the reaction mixtures was saponified with aqueous sodium hydroxide and the resulting cooled solution was saturated with carbon dioxide. This served to precipitate 0.13 gm. of unaltered estrone, which was removed by filtering the suspension through moist fullers' earth. The filtrate was acidified and warmed, and the precipitate (0.14 gm.) was collected. This "lactone" fraction was acetylated and the product was taken up in ether and washed successively with dilute hydrochloric acid, water, and sodium bicarbonate. The last removed 0.01 gm. of acidic material, which was discarded. The neutral fraction crystallized very slowly from aqueous methanol to give 0.09 gm. of solid melting at 124–135°. After further recrystallization, this melted at 127–130° and no pure substance IV could be obtained from it. The Allen-Doisy unit was found to be about 10  $\gamma$ .

*Oxidation of Estrone Acetate with Hydrogen Peroxide in Acetic Acid*—To a solution containing 2.5 gm. of estrone acetate (m.p. 125–126°) in 20 ml. of glacial acetic acid were added 13.5 ml. of 30 per cent aqueous hydrogen peroxide. The solution was allowed to stand in the dark at 35° for 60 hours. The reaction mixture was then gradually diluted with water until the crystallization of the product was complete. In several such experiments, the crude product melted at 144–148° and was obtained in 57 to 63 per cent yield.

The aqueous mother liquor, which contained the alteration products of about 40 per cent of the estrone acetate used, was cooled, treated with sulfur dioxide, and concentrated at 40° under diminished pressure. The residue, part of which was sparingly soluble in water, provided no additional crystalline material.

In many orienting oxidation experiments the proportion of the reactants and the reaction temperature were varied within wide limits in establishing the optimum conditions described above.

*Estranolactone (IIIb)*—The crude product (6.94 gm.) from several estrone acetate oxidations was suspended in 150 ml. of 20 per cent methanol containing 25 to 30 ml. of 10 per cent sodium hydroxide and heated on the steam bath until the solid dissolved (1 to 2 hours). The resulting solution was diluted with 75 ml. of water, cooled to 15°, and saturated with carbon dioxide (neutrality to phenolphthalein), the latter precipitating 0.32 gm.

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the thermometer stem or with a thermometer graduated for partial stem immersion. Melting points marked "block" were determined in a preheated aluminum block and were taken as the temperature at which the compound actively melted during a period of 1 or 2 minutes. Samples for analysis were dried at 110° and 0.05 mm. over phosphorus pentoxide, except as otherwise noted. Microanalyses were performed by Dr. Robert T. Dillon and staff of the Analytical Division of G. D. Searle and Company, whose assistance in this work the author gratefully acknowledges.

of unaltered estrone. This was removed by suction filtration, with a small amount of fullers' earth as a filter aid, and the filtrate was strongly acidified with hydrochloric acid and warmed to 60–65° to promote the lactonization of the precipitated acid. After about 30 minutes, the suspension was cooled and the finely divided, crystalline lactone was collected. This material (5.7 gm.), melting at about 330° (block), served in most instances for the preparation of the functional derivatives described below. It is very sparingly soluble in most organic solvents and was purified by recrystallization from cyclohexanone or methyl cellosolve, from which it separated in the form of small prisms melting at 339° (block). Westerfeld (4) reported a melting point of 335–340° for his lactone. The sample for analysis was dried at 135°.

$C_{18}H_{22}O_2$ .	Calculated.	C 75.49, H 7.74
	Found.	" 75.23, " 7.72
		" 75.51, " 7.83

*Esters of Estrololactone.* (a) *Acetate (IV)*—The acetylation of 5.7 gm. of the precipitated IIIb was accomplished by heating it with 10 ml. of acetic anhydride in 15 ml. of pyridine for 2 hours on the steam bath. The product was decolorized by dissolving it in carbon tetrachloride and filtering the dried solution through a 2 × 8 cm. column of 80 mesh Alorco-A alumina. The colorless, crystalline residue from the eluate and washings crystallized from methanol in heavy, diamond-shaped crystals (5.51 gm.) melting at 149–150.5°.  $[\alpha]_D^{24} = +42^\circ$  ( $c = 0.277$ , chloroform). Westerfeld (4) reported 143.5–145° for the acetate of III.

$C_{20}H_{24}O_4$ .	Calculated.	C 73.14, H 7.37
	Found.	" 73.47, " 7.48
		" 73.28, " 7.49

(b) *Propionate (V)*—A suspension of 0.20 gm. of precipitated IIIb in 1 ml. of propionic anhydride and 1 ml. of pyridine was heated for 2 hours. The product crystallized from aqueous methanol in the form of needles melting at 146–148.5°.

$C_{21}H_{26}O_4$ .	Calculated.	C 73.66, H 7.65
	Found.	" 73.72, " 7.70
		" 73.93, " 7.80

(c) *Benzoate (VI)*—The benzylation of 0.20 gm. of IIIb with 1 ml. of benzoyl chloride in 4 ml. of pyridine and recrystallization of the product from ethanol or acetone formed small needles which melted at 241–244°. The sample for analysis was dried at 135°.

$C_{25}H_{28}O_4$ .	Calculated.	C 76.90, H 6.71
	Found.	" 77.19, " 6.78
		" 76.91, " 6.74

*Methyl Ether of Estroloactone (VII)*—430 mg. of IIIb were saponified by refluxing the suspension in a solution of 0.17 gm. of potassium hydroxide in 20 ml. of *n*-propanol until the solid completely dissolved. After the alcohol was removed under diminished pressure, the residue in 10 ml. of water was shaken at 50–60° with several 0.2 ml. portions of dimethyl sulfate, the solution being maintained alkaline to phenolphthalein with 10 per cent potassium hydroxide. The separating solid began to turn yellow and gather into lumps after 1.0 ml. of dimethyl sulfate had been added. At this stage, the reaction mixture was acidified and the solid was saponified with aqueous methanolic potassium hydroxide. The hot, methanol-free solution was then strongly reacidified and the product was crystallized from aqueous methanol to give 0.38 gm. of small, flat needles melting at 167–172°. Repeated recrystallization served to raise the melting point to 172.5–174°. Westerfeld (4) reported 166–168° for the methyl ether of III.

$C_{19}H_{24}O_3$ .	Calculated.	C 75.97, H 8.05
	Found.	" 75.94, " 8.00
		" 76.06, " 7.92

The methyl ether was prepared from IIIb (0.22 gm.) with methyl iodide (5 ml.) containing silver oxide added in three 0.2 gm. portions during a refluxing period of about 30 hours. As the lactone slowly dissolved, a few lumps of Drierite were added to the boiling suspension. The crude crystalline product (0.15 gm.) melted at 167–171.5° and this melting point was not depressed by admixture with pure material prepared by the alkali-dimethyl sulfate procedure.

*Estrolic Acid (VIII)*—250 mg. of IV were saponified by heating with an excess of dilute aqueous sodium hydroxide and the cooled (15°) solution was slowly acidified with vigorous stirring to about pH 5 (faint darkening of Congo red) with hydrochloric acid. The solid acid was collected and recrystallized by dissolving it in warm acetone, removing by filtration a small amount of the sparingly soluble IIIb, and diluting the concentrated filtrate with water. The acid (0.15 gm.) separated in the form of small plates which melted with effervescence at 225° (block). If the melting point was determined in the usual way, the acid gradually lactonized without sintering and melted at about 330°. The sample for analysis was dried at 25°.

$C_{13}H_{24}O_4$ .	Calculated.	C 71.03, H 7.95
	Found.	" 70.9, " 8.08
		" 71.2, " 8.22

*Methyl Estrolate (XI)*—This ester was prepared by treating VIII with an excess of cold, ethereal diazomethane and crystallizing the product from aqueous methanol. The glistening needles which separated became

opaque on standing in a dry atmosphere and then melted at 95–97°. The sample for analysis was dried at 65° for 1 hour.

$C_{18}H_{26}O_4$ .	Calculated.	C 71.67, H 8.23
$C_{18}H_{26}O_4 \cdot CH_3OH$ .	"	" 68.54, " 8.63
	Found.	" 68.8, " 8.66
		" 68.8, " 8.51

When this was dried for 8 hours at 60–65°, the analytical values were C 71.2, H 8.34.

*Hydrolysis of XI*—A mixture of 0.10 gm. of pure XI and 0.07 gm. of potassium bicarbonate in 6 ml. of methanol and 4 ml. of water was refluxed on the steam bath for 10 minutes. Crystalline material began to separate during the first few minutes of boiling. The suspension was evaporated in a stream of air, the residue was shaken with 20 ml. of warm water, and the solid was collected. The product melted at 336–338° (block) and weighed 0.085 gm.

*Sodium Estrolate (IX)*—1.4 gm. of IV were saponified by heating with a mixture of 15 ml. of 10 per cent sodium hydroxide and 15 ml. of 10 per cent methanol, after which the solution was diluted with 50 ml. of water. This was cooled, saturated with carbon dioxide, and concentrated below 40° to yield the crude crystalline sodium salt (two crops from the concentrated sodium bicarbonate-sodium acetate solution). After purification by recrystallization from ethanol, there were obtained 1.35 gm. of fine needles (clusters) melting at 225° (block). The sample for analysis was dried at 25°.

$C_{18}H_{22}O_4Na \cdot 2H_2O$ .	Calculated.	C 59.65, H 7.51, Na 6.35
	Found.	" 59.5, " 7.92, " 6.21

Although the dihydrate appears to be stable, the salt dried at 100° is highly hygroscopic and quickly reverts to the dihydrate. In attempting to obtain the anhydrous material, Dr. Dillon reported that the carbon values approached those calculated for the hemihydrate, but were not increased further by prolonged heating *in vacuo* at 100°.

*Estrolic Acid Methyl Ether (X)*—The methyl ether lactone (VII) was saponified with aqueous methanolic sodium hydroxide and the methanol-free solution was carefully acidified, as in the preparation of VIII. The acid crystallized from aqueous acetone in the form of elongated hexagonal tablets melting at 135–136° with effervescence. The sample for analysis was dried at 25°.

$C_{18}H_{26}O_4$ .	Calculated.	C 71.67, H 8.23
	Found.	" 71.44, " 8.07
		" 71.91, " 8.10

The *methyl ether methyl ester* (XII) was obtained by treating X with an excess of cold ethereal diazomethane and crystallizing the product from aqueous methanol. It formed glistening leaves which melted at 64.5–65.5° and, after drying at 65°, became opaque and then melted at 77.5–78.5°.

$C_{26}H_{28}O_4$ . Calculated.	C 72.26, H 8.49
Found (77.5–78.5°).	" 72.2, " 8.56
	" 72.5, " 8.64
Calculated. $OCH_3$	18.67; after KOH hydrolysis, 9.34
Found.	" 18.40, 18.42; after KOH hydrolysis, 9.40

*Benzoylation of XI*—In the attempted benzoylation of XI, a solution containing 0.56 gm. of the ester in 5 ml. of pyridine was heated with 2 ml. of benzoyl chloride for  $2\frac{1}{2}$  hours. After treatment with water, the reaction mixture was extracted with ether and the ethereal solution was washed successively with hydrochloric acid, water, sodium bicarbonate, and saturated sodium chloride solution. The residue from the ether solution was leached with warm methanolic acetone and the insoluble material (0.095 gm.), melting at 238–243°, was collected. By further recrystallization of the dissolved material from aqueous acetone and benzene-cyclohexane, 0.21 gm. of a faintly yellow, nodular solid was obtained, while the remainder of the product was non-crystalline. The combined solid crystallized readily from acetone in the form of very small needles (0.27 gm.) melting at 240–244°; mixed with the lactone benzoate (VI), this product melted at 241–244°.

The non-crystalline fraction was subjected to alkaline hydrolysis and the solution was acidified and warmed. The yellow gum which formed was leached with 80 per cent acetone, some insoluble solid was separated, and the solution was allowed to stand for several days. The finely divided solid, which was collected in several crops, was recrystallized from aqueous acetone to form a mixture of sparingly soluble powder and well formed blades. The latter (0.12 gm.), which were separated mechanically, melted at 167–171°. This fraction, after acetylation, failed to react with diazomethane and was recrystallized from ether-acetone, from which it separated as heavy blades, m.p. 168–172°. In an attempt to purify the "acetate" for analysis, the substance was so strongly adsorbed on the alumina that it could not be chromatographed. By leaching the alumina with hot 80 per cent acetic acid, about half (0.065 gm.) of the material was recovered and this was set aside for further study. When the benzoylation was conducted at 23° for 24 hours, the character of the products was the same.

*Attempted Dehydration of XI*—In several attempts to effect the dehydration of XI, the ester was treated (a) with acetic anhydride-pyridine and the non-crystalline product was then heated on the steam bath for 1 hour

with phosphorus oxychloride in pyridine; (b) with 98 to 100 per cent formic acid for 3 hours on the steam bath or by refluxing for 1 hour; or (c) with iodine in refluxing toluene or xylene. In all instances the major product was the lactone IIIb or its esters.

The Clemmensen reduction of the lactone acetate (IV, 0.36 gm.) with amalgamated zinc in boiling acetic-hydrochloric acid produced 0.01 gm. of IIIb and 0.27 gm. of acidic material. The acids crystallized partially from aqueous acetone in small needles, melting in the range 205–225°. The resolution of this mixture by the chromatographic separation of the acetylated methyl esters will be described in a later paper.

#### SUMMARY

The oxidation of estrone acetate with hydrogen peroxide in acetic acid is reported to form, in 57 to 63 per cent crude yield, the lactone acetate of estrolic acid. The partial characterization of this acid is described. The suspected non-identity of certain estrolic acid derivatives with similar products previously reported by Westerfeld (4) is discussed.

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# STEROID LACTONES

## II. THE PREPARATION OF ISOANDROLIC ACID AND RELATED COMPOUNDS\*

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The oxidation of estrone acetate with aqueous hydrogen peroxide in acetic acid, to form the lactone acetate (I) of estrolic acid (II), has been described in a previous report (1) of this series. The presence of a lactone ring in I and its derivatives was demonstrated, but the structure and configuration of the compounds were not established. Estrolic acid and certain of its derivatives have been found to possess little estrogenic activity and to be capable of inhibiting<sup>1</sup> the secretion of gonadotropic hormone by the pituitary gland. The discovery of these interesting physiological properties of the estrone oxidation products has led us to the preparation of analogous lactonic compounds from 17-keto steroids of the androgenic type.

The present report describes the preparation of several new lactones which are, or may be considered to be, derived from androsterone, isoandrosterone, dehydroisoandrosterone, testosterone, and androstan-3,17-dione.

An alcoholic solution of dehydroisoandrosterone acetate (III) was reduced at atmospheric pressure, with 10 per cent palladinized norit as catalyst, to form isoandrosterone acetate (IV) in about 80 per cent yield. None of the isomeric etiocholan-3( $\beta$ )-ol-17-one acetate was isolated in a pure state. Similar results were obtained when the reduction was conducted in acetic acid with Adams' platinic oxide or in alcohol with palladous chloride. These observations are in contrast with those of Reichstein and Lardon (2) who, using platinic oxide in acetic acid, obtained IV, m.p. 103–104°, and etiocholan-3( $\beta$ )-ol-17-one acetate, m.p. 157–159°, in respective yields of 36 and 4 per cent. Further, in the present work, IV crystallized almost exclusively as the hitherto unreported polymorph, melting at 116.5–117°, rather than as the known form, m.p. 104.5–105°.

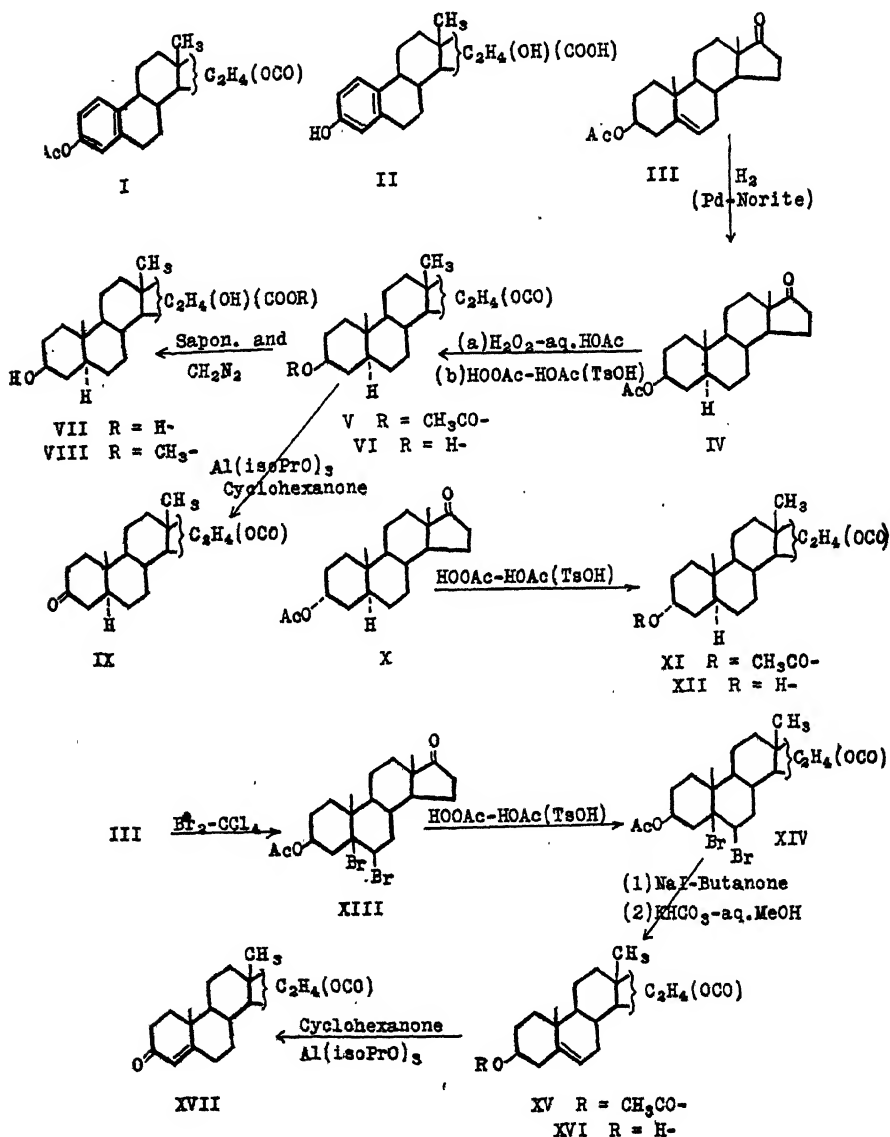
Isoandrosterone acetate was oxidized with aqueous hydrogen peroxide

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<sup>1</sup> A full report of the physiological studies will be made in a later paper.



in acetic acid at 35° for 53 hours to the corresponding lactone (V) in about 80 per cent yield. An improvement in the oxidation reaction was achieved



by using dilute peracetic acid in glacial acetic acid containing a small amount of *p*-toluenesulfonic acid ( $\text{TsOH}$ ). With this reagent the yield of nearly pure V was 88 to 92 per cent after 24 hours at 35°. Saponification

of V and subsequent relactonization with acid transformed it into the hydroxy lactone (VI), to which the name, isoandrololactone, is assigned. The presence of a lactone ring was demonstrated by converting VI into the dihydroxy acid (VII, isoandrollic acid) and the latter, in turn, into the dihydroxy methyl ester (VIII, methyl isoandrolate).

Isoandrololactone was oxidized with cyclohexanone in the presence of aluminum isopropoxide to the corresponding 3-keto lactone (IX), m.p. 166–167.7°. This compound ( $C_{19}H_{28}O_3$ ) is not identical with the "saturated"<sup>2</sup> keto lactone, m.p. 252°, the isolation of which from the non-phenolic fraction of mare pregnancy urine has been reported by Heard (3) and by Jacobs and Laqueur (4).

By use of peracetic acid as described above, androsterone acetate (X) was oxidized to the acetoxy lactone (XI) in 79 per cent yield. This was then hydrolyzed and relactonized to form XII (andrololactone).

In order to prepare the lactonic analogue (XVII) of testosterone, dehydroisoandrosterone acetate (III) was employed as the starting material. The latter was brominated in carbon tetrachloride and the resulting 5,6-dibromide (XIII), on oxidation with peracetic acid, afforded the lactone dibromide (XIV) in 86 to 93 per cent yield. The debromination of XIV by means of a refluxing solution of sodium iodide in butanone led to the unsaturated acetoxy lactone (XV) and, by subsequent hydrolysis, to the corresponding unsaturated hydroxy compound (XVI, dehydroisoandrololactone). Finally, oxidation of the latter with cyclohexanone and aluminum isopropoxide provided the  $\alpha,\beta$ -unsaturated ketone, testololactone (XVII).<sup>3</sup> The ultraviolet absorption spectrum of XVII showed a maximum at 238  $m\mu$ , with  $\log \epsilon = 4.23$ .

The physiological properties of these lactones will be reported in detail in a later paper. They all lack any demonstrable androgenic activity but definitely affect the pituitary. Thus, isoandrololactone stimulates pituitary growth and the formation of pituitary gonadotropin in male and female rats.

Work in this laboratory is continuing on the elucidation of the structure and configuration of these and other lactonic oxidation products of keto steroids.

<sup>2</sup> The analytical data reported by both Heard and by Jacobs and Laqueur correspond closely to the empirical formula  $C_{19}H_{28}O_3$ , which is not in accord with a saturated tetracyclic structure. It would appear therefore that the compound, m.p. 252°, from mare urine must either contain an unreactive ethylenic bond or be isomeric with IX and contain 2 more hydrogen atoms.

<sup>3</sup> Testololactone may be isomeric with the keto lactone of Heard (3), but the two compounds are not identical.

*Reduction of Dehydroisoandrosterone Acetate*—Dehydroisoandrosterone acetate<sup>3</sup> (III, 2.00 gm., m.p. 168–169°) was reduced at atmospheric pressure in 30 ml. of 95 per cent ethanol containing 0.5 gm. of 10 per cent palladinized norit catalyst. The theoretical amount of hydrogen was absorbed in 5 hours. The mixture was filtered, the filtrate concentrated *in vacuo* to a syrup, and the isoandrosterone acetate allowed to crystallize from a solution of the syrup in neohexane. Several crops, totaling 1.64 gm. (82 per cent) and melting in the range 113–115.5°, were obtained and recrystallized from neohexane or aqueous methanol. The final melting point was 116.5–117°.

In another experiment, with platinum oxide in glacial acetic acid, the yield of IV, m.p. 114–117°, was 65 per cent. During subsequent recrystallization from neohexane, the compound separated either in this form (m.p. 116.5–117°) or as the modification melting at 104.5–105°. When a large proportion of the latter was mixed with a few crystals of the former, the melting point of the mixture was 116.2–116.8°, showing that the two products are polymorphic forms of the same compound. During the purification of the product from this reduction, there was isolated 0.06 gm. of material which, after recrystallization from pentane, melted at 143–151°. This may have contained some etiocholan-3( $\beta$ )-ol-17-one acetate (m.p. 157–159°), but it was not further studied.

In a separate experiment, III was reduced in 95 per cent ethanol with palladous chloride as the catalyst. The crude product was obtained in several crops (84 per cent yield), melting in the ranges 100–103.5° and 109–115.2°.

*Isoandrolactone Acetate (V). Method A, Aqueous Hydrogen Peroxide in Acetic Acid*—A solution of isoandrosterone acetate (0.25 gm., 0.00075 mole) in 2.0 ml. of glacial acetic acid and 1.5 ml. (about 0.12 mole) of 28 per cent aqueous hydrogen peroxide was allowed to stand in the dark at 35° for 53 hours. A large volume of water was added, dropwise until crystallization began, to precipitate 0.21 gm. (80 per cent) of crude lactone, m.p. 149.5–153°. This was then saponified by refluxing its solution in 3 ml. of

<sup>4</sup> All melting points were taken of samples in an open capillary, with a totally immersed thermometer, unless indicated otherwise. Microanalyses were performed by Dr. Robert T. Dillon and staff of the Analytical Division of G. D. Searle and Company, and by Mr. William Saschek of the Department of Biochemistry, College of Physicians and Surgeons, Columbia University. Their cooperation is gratefully acknowledged. All samples for analysis were dried over phosphorus pentoxide at 80° and 0.05 mm.

<sup>5</sup> We are indebted to Dr. Erwin Schwenk of the Schering Corporation who generously contributed the dehydroisoandrosterone acetate used in this investigation.

methanol and 1.1 ml. of 10 per cent methanolic potassium hydroxide for  $1\frac{1}{2}$  hours. The solution was concentrated *in vacuo* to dryness, the residue dissolved in water, and the solution extracted with chloroform to remove about 0.005 gm. of neutral, yellow oil. It was then strongly acidified with 3 N hydrochloric acid and the resulting suspension was heated for 1 hour on the steam bath to induce lactonization. The solid was removed by ether extraction and the dried extract was concentrated to a yellow syrup. This syrup was acetylated with 2 ml. of acetic anhydride and 5 drops of pyridine by heating for 1 hour on the steam bath. The mixture was concentrated *in vacuo* and the residual solid was crystallized twice from benzene-neohexane to produce transparent blades melting at 158–159.5°.

$C_{21}H_{32}O_4$ . Calculated, C 72.37, H 9.26; found, C 72.58, H 9.10

*Method B, Peracetic Acid in Acetic Acid*—Isoandrosterone acetate (0.274 gm.) in 2.0 ml. of glacial acetic acid was oxidized with 5.0 ml. of a solution of peracetic acid in glacial acetic acid (5) (0.00125 mole per ml.) in the presence of 20 mg. of additional *p*-toluenesulfonic acid. The oxidation was conducted in the dark at 35° for 23 hours. The crude lactone (0.252 gm., 88 per cent), m.p. 156–158.5°, was obtained as described above.

In several similar oxidations, the yield was 89 to 92 per cent and one recrystallization of this material served to raise the melting point to 158.5–159.5°.

*Isoandrololactone (VI)*—Isoandrololactone acetate (V, 0.10 gm.) in 4 ml. of methanol and 0.5 ml. of water was saponified by refluxing for 1 hour with 0.55 ml. of 10 per cent methanolic potassium hydroxide. The solution was acidified with 0.55 ml. of 3 N hydrochloric acid, diluted with 5 ml. of water, and heated on the steam bath for 1 hour in a current of nitrogen. The crude, precipitated lactone (0.08 gm., 91 per cent), melted at 162.5–166.5°, and this melting point was raised to 169.7–169.9° by recrystallization from ethyl acetate-neohexane.

$C_{19}H_{30}O_2$ .	Calculated.	C 74.48, H 9.87
	Found.	" 74.15, " 9.74
		" 74.39, " 9.80

*Isoandrololic Acid (VII)*—Isoandrololactone (0.29 gm.) was heated under nitrogen for  $1\frac{1}{2}$  hours on the steam bath with 1.5 ml. of 10 per cent aqueous sodium hydroxide and 15 ml. of water. The solution was cooled to room temperature and a trace of insoluble matter was removed. The filtrate was then cooled to 15° and acidified with 1.3 ml. of 3 N hydrochloric acid to about pH 3. The precipitate of crude acid weighed 0.28 gm. (91 per cent) and melted at 166.5–167.5° with decomposition. The pure acid (72 per cent yield) was obtained in the form of blades melting at 170.5–

171.3°, with decomposition (bath preheated to 165°), by recrystallization from ethyl acetate.

$C_{19}H_{32}O_4$ .	Calculated.	C 70.32, H 9.94
	Found.	" 70.16, " 9.60
		" 70.23, " 9.84

*Methyl Isoandrolate (VIII)*—A large excess of cold, ethereal diazomethane was added in portions to a suspension of 0.10 gm. of VII in 20 ml. of ice-cold ether. The acid readily reacted and dissolved. The excess diazomethane was removed by gentle warming, and the solution was filtered and evaporated to dryness. The residual oil quickly solidified (0.10 gm.) and remelted at 104–106.4°. The pure ester, m.p. 118.4–118.9°, was achieved in the form of clusters of blades by recrystallization from ethyl acetate-neohexane. The yield of purified solids, with a total melting range of 115–118.9°, was 85 per cent.

$C_{20}H_{34}O_4$ .	Calculated.	C 70.96, H 10.12, $OCH_3$ 9.17
	Found.	" 71.17, " 10.46, " 9.08
		" 70.97, " 10.25

*Oxidation of Isoandrololactone to 3-Ketolactone (IX)*—Isoandrololactone (0.33 gm.) was oxidized to the corresponding 3-keto lactone by refluxing for 6 hours with 0.35 gm. of aluminum isopropoxide in 3.5 ml. of cyclohexanone and 10 ml. of dry toluene. The cooled reaction mixture was diluted with ether and washed successively with 3 N hydrochloric acid, water, saturated sodium bicarbonate, and water. The dried ethereal solution was then concentrated and the residual oil allowed to crystallize from 25 ml. of pentane. The crude substance IX (0.16 gm., 49 per cent) melted at 159–164.5° and this was raised to 166–167.7° by recrystallization from ethyl acetate-neohexane, from which the keto lactone separated in rosettes of thick blades.

$C_{19}H_{32}O_5$ .	Calculated, C 74.97, H 9.27; found, C 74.82, H 9.46
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The yield above is not optimum, for a longer refluxing period would undoubtedly raise it. For example, only a trace of IX was produced after 2 hours.

*Andrololactone Acetate (XI)*—Androsterone acetate (0.17 gm., m.p. 165–165.5°) was oxidized to the lactone with peracetic acid as described above. The crude, water-precipitated lactone acetate weighed 0.14 gm. (79 per cent) and melted at 176.2–180.7°. This was recrystallized from ethyl acetate-neohexane to form needles melting at 184.1–185.4°.

$C_{21}H_{32}O_4$ .	Calculated, C 72.37, H 9.26; found, C 72.58, H 9.37
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*Andrololactone (XII)*—The lactone acetate (XI, 0.12 gm.) was converted into the hydroxy lactone in the manner described above for the iso compound. The crude product (0.095 gm., 90 per cent, m.p. 213.5–222.5°) crystallized from ethyl acetate in the form of blades which melted at 229.6–232° (bath preheated to 225°). On the Fisher-Johns block (preheated to 225°), the melting point was 237–237.5°.

$C_{19}H_{26}O_3$ . Calculated, C 74.48, H 9.87; found, C 74.56, H 9.84

*5,6-Dibromo-3(β)-acetoxyandrostan-17-one (XIII)*—0.5 gm. of dehydroisoandrosterone acetate was brominated in 10 ml. of cold carbon tetrachloride by the dropwise addition of 6.35 ml. of the same solvent containing 0.26 gm. of bromine. The solution became decolorized after 20 minutes stirring at 2–5°. It was then concentrated *in vacuo* and the solid residue was crystallized from benzene-neohexane or ethyl acetate-neohexane, affording 0.70 gm. (94 per cent) of colorless dibromide melting at 161.4–162.4°, with decomposition. Further recrystallization raised the melting point to 162.9–163.2°, with decomposition (bath preheated to 159°).

$C_{21}H_{30}O_3Br_2$ . Calculated. C 51.45, H 6.17, Br 32.60  
Found. " 51.57, " 5.83, " 32.20

In another preparation, the yield was reduced to 57 per cent when the reaction was conducted at room temperature with no external cooling.

*Lactone (XIV) from 5,6-Dibromo-3(β)-acetoxyandrostan-17-one*—The above dibromide was oxidized to the corresponding lactone with peracetic acid in the usual manner. The yield of crude, precipitated solid (m.p. 170.3–170.7°, with decomposition) was 86 to 93 per cent. Rosettes of needles melting at 170.5–170.9°, with decomposition (bath preheated to 169°), were obtained by recrystallization from ethyl acetate-neohexane.

$C_{21}H_{30}O_4Br_2$ . Calculated. C 49.82, H 5.97, Br 31.57  
Found. " 49.61, " 6.02, " 31.25

*Dehydroisoandrololactone Acetate (XV)*—A solution of the dibromo lactone (XIV, 0.81 gm.) and 2.0 gm. of sodium iodide in 50 ml. of butanone<sup>6</sup> was refluxed for 5 hours. The solution was cooled and shaken with aqueous sodium bisulfite to reduce the iodine. The ketone layer was concentrated *in vacuo*, the residue was dissolved in ethyl acetate, and the solution washed with aqueous sodium chloride solution, dried, and concentrated. Diluting the residue with benzene-neohexane induced the separation of the crude, crystalline product XV (0.47 gm., 84 per cent), melting at 180–181°. Fur-

<sup>6</sup> We are indebted to the Shell Chemical Corporation for a generous gift of this ketone.

ther recrystallization raised the melting point to 183–185° (bath preheated to 180°).

$C_{21}H_{30}O_4$ .	Calculated.	C 72.81, H 8.73
	Found.	" 72.96, " 8.85
		" 72.92, " 8.52

*Dehydroisoandrololactone* (XVI)—The lactone acetate (XV, 0.87 gm.), dissolved in 25 ml. of methanol, was hydrolyzed by refluxing for 4 hours with a solution of 1.8 gm. of potassium bicarbonate in 13 ml. of water. The warm reaction mixture was acidified by the addition of 9 ml. of 3 N hydrochloric acid and heated on the steam bath for 1 hour under a stream of nitrogen. It was then diluted with 50 ml. of water, cooled to 15°, and the precipitated hydroxy lactone (0.75 gm., 98 per cent) collected. The melting point (235–241.5°) of the crude product was raised to 238–242.2° (bath preheated to 232°) by recrystallization from ethyl acetate.

$C_{19}H_{28}O_3$ .	Calculated,	C 74.97, H 9.27; found, C 75.10, H 9.11
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*Testololactone* (XVII)—A mixture of 0.3 gm. of XVI, 0.3 gm. of aluminum isopropoxide, 3 ml. of cyclohexanone, and 9 ml. of dry toluene was refluxed for 48 hours. The reaction mixture was cooled and diluted with ether, and the suspension was washed successively with 3 N hydrochloric acid, aqueous sodium bicarbonate, and saturated sodium chloride solution. The dried ether solution was concentrated and the residue was heated at 0.5 mm. and 90° to remove most of the solvents. The residue in a small volume of ethyl acetate was then diluted with neohexane to effect the separation of 0.27 gm. (91 per cent) of crystalline lactone melting at 195.5–205.5°. By repeated recrystallization, rosettes of needles were obtained which melted at 205.5–207°.

$C_{19}H_{28}O_3$ .	Calculated.	C 75.46, H 8.67
	Found.	" 75.54, " 8.51
		" 75.37, " 8.37

The ultraviolet absorption spectrum of a solution ( $6.59 \times 10^{-5}$  M, 1 cm. path) of XVII in absolute ethanol showed a maximum at 238 m $\mu$ ,  $\log \epsilon = 4.23$ .

#### SUMMARY

The oxidation of certain 17-keto steroids to their corresponding Ring D lactones by means of aqueous hydrogen peroxide in acetic acid or by peracetic acid in glacial acetic acid is described. These lactones are, or may be considered to be, derived from androsterone, isoandrosterone, dehydroisoandrosterone, testosterone, and androstan-3,17-dione. Neither

of the last two lactones is identical with the keto lactone, m.p.  $252^{\circ}$ , reported by Heard and by Jacobs and Laqueur.

A hitherto unobserved polymorphic form of isoandrosterone acetate, m.p.  $116.5\text{--}117^{\circ}$ , is described.

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## STEROID LACTONES

### III. THE PREPARATION OF BISDEHYDROESTROLIC ACID AND ITS DERIVATIVES\*

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Previous papers (1, 2) from this laboratory have reported the preparation of lactones by the peroxide oxidation of a number of 17-keto steroids. In these oxidations two reaction media were employed, one consisting of a mixture of aqueous hydrogen peroxide and acetic acid and the other of a solution of peracetic acid in glacial acetic acid containing a small amount of *p*-toluenesulfonic acid.

It was recognized early in this work that the small equilibrium concentration (3) of peracetic acid present in the aqueous hydrogen peroxide-acetic acid mixtures first used (1) might be the active oxidizing agent. Indeed, the subsequent use (2) of non-aqueous peracetic acid solutions in the high yield oxidations of 17-keto steroids of the androgenic hormone type provided confirmation of this idea. However, when attempts were made to apply the improved procedure in the oxidation of estrone and equilenin, much of the steroid ketone was converted to amorphous material and no enhancement of the yield of lactone was observed. After several trial experiments it became clear that, whereas the saturated 17-keto steroids were smoothly oxidized with peracetic acid in less than 20 hours at 35°, those of the aromatic type required a much lower temperature and longer reaction time.

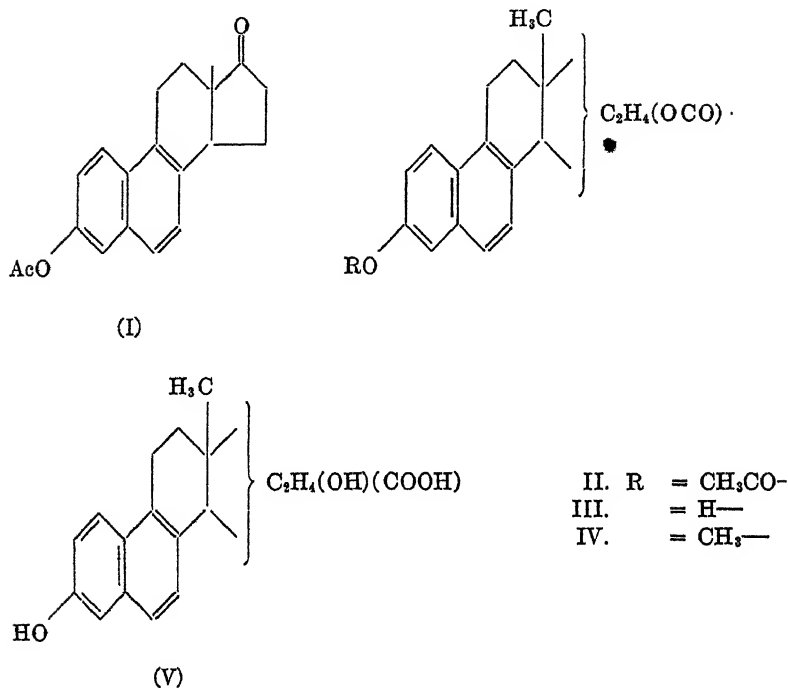
This paper describes the preparation of bisdehydroestrolic acid derivatives from equilenin, as well as the improved oxidation method as applied to estrone acetate.

In the initial experiments, equilenin acetate (I) was oxidized at 35° with hydrogen peroxide in acetic acid, forming in low yield the lactone acetate (II) contaminated with unaltered starting material. When peracetic acid was used, the yield of nearly pure product II obtained at 35°

\* The work described in this paper was supported by a grant from G. D. Searle and Company.

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was about 20 per cent and this was increased about 3-fold when the reaction temperature was lowered to 10°.



The saponification and relactonization (acidification with heating) of II afforded the hydroxy lactone (III). When the saponification mixture was acidified to about pH 5 at a low temperature, bisdehydroestrollic acid (V) was produced.

The methyl ether (IV) of III was prepared by the saponification of II followed by treatment of the warm alkaline solution with dimethyl sulfate.

This series of reactions demonstrates the lactonic nature of II, III, and IV, and shows that the structure of bisdehydroestrollic acid is similar to that of the previously reported oxidation products of other 17-keto steroids.

In the preparation of relatively large quantities of estrollic acid, it was discovered that the above oxidation procedure with peracetic acid at 10° led to the production of estrololactone acetate in 80 per cent yield, compared with about 60 per cent by the older (1) method.

Work in this laboratory is continuing on the oxidation of steroid ketones and on the elucidation of the structure and configuration of the products obtained.

EXPERIMENTAL<sup>1</sup>

*Bisdehydroestrololactone Acetate (II). Method A, Oxidation of Equilenin Acetate with Aqueous Hydrogen Peroxide in Acetic Acid*—A solution containing 0.62 gm. of equilenin acetate<sup>2</sup> (m.p. 154.5–157°) in 6 ml. of acetic acid and 1.5 ml. of 30 per cent aqueous hydrogen peroxide was allowed to stand at 35° for 60 hours. When the reaction mixture was diluted with water, a yellow, partially crystalline solid separated. In several such experiments, the yield of this crude product was 30 to 40 per cent and the melting point, after one recrystallization from aqueous methanol, was 142–147°. When 0.72 gm. of this material was hydrolyzed with aqueous methanolic potassium hydroxide and the cold, methanol-free solution was saturated with carbon dioxide, 0.23 gm. of equilenin (m.p. 250–254°, after one recrystallization from methanol) was precipitated. The filtrate was strongly acidified and warmed, causing the separation of 0.35 gm. of sparingly soluble, brown solid. This was acetylated with acetic anhydride and pyridine and the product recrystallized from aqueous methanol to form fine, colorless needles, m.p. 155–157.5°.

$C_{20}H_{20}O_4$ . Calculated, C 74.05, H 6.22; found, C 73.68, H 6.29

*Method B, Oxidation of Equilenin Acetate with Peracetic Acid in Glacial Acetic Acid*—A solution of 1.0 gm. of equilenin acetate in 20 ml. of glacial acetic acid containing 0.02 mole of peracetic acid and about 20 mg. of *p*-toluenesulfonic acid was held at 10° for 100 hours. The reaction mixture was gradually diluted with water until crystallization of the product was complete. This provided 0.58 gm. (55 per cent) of colorless needles, m.p. 156°, after a single recrystallization from methanol.

$C_{20}H_{20}O_4$ . Calculated. C 74.05, H 6.22

Found. " 74.3, " 6.12

" 74.0, " 6.26

When the above reaction was conducted at 35°, the yield of lactone was only 20 to 25 per cent.

<sup>1</sup> All melting points were determined in open capillaries with total immersion of the thermometer stem or with a thermometer graduated for partial stem immersion. Melting points marked "block" were determined in a preheated aluminum block and were taken as the temperature at which the compound actively melted during a period of 1 or 2 minutes. Samples for analysis were dried at 110° and 0.05 mm. over phosphorus pentoxide except as otherwise noted. Microanalyses were performed by Dr. Robert T. Dillon and staff of the Analytical Division of G. D. Searle and Company, whose assistance in this work the authors gratefully acknowledge.

<sup>2</sup> We are indebted to Parke, Davis and Company, Detroit, Michigan, for a supply of equilenin.

*Bisdehydroestrololactone (III)*—The lactone acetate (II, 0.36 gm., m.p. 154–155.5°) was suspended in 25 ml. of 2 per cent aqueous sodium hydroxide and heated on the steam bath for 2½ hours. The hot solution was acidified with excess hydrochloric acid and allowed to cool slowly. The pale pink, crystalline product weighed 0.30 gm. and, after recrystallization from methyl cellosolve, melted at 292° (block).

$C_{18}H_{18}O_2$ .	Calculated.	C 76.57, H 6.43
	Found.	" 76.5, " 6.43
		" 76.7, " 6.43

*Bisdehydroestrololactone Methyl Ether (IV)*—The lactone acetate (II, 0.35 gm.) was saponified as above with dilute sodium hydroxide and the solution at 50–60° was shaken with 1.0 ml. of dimethyl sulfate while the reaction mixture was kept alkaline to phenolphthalein with 10 per cent sodium hydroxide. The suspension was acidified and the precipitated product was saponified with hot aqueous methanolic sodium hydroxide. After removal of the methanol, the hot alkaline solution was strongly acidified and the crude methyl ether was collected. By recrystallization of this material from methanol, there was obtained 0.10 gm. of glistening blades, m.p. 196–199°. This melting point was raised to 197.3–199.5° by further recrystallization.

$C_{19}H_{20}O_2$ .	Calculated.	C 77.00, H 6.80
	Found.	" 76.9, " 6.92
		" 76.8, " 6.71

*Bisdehydroestrololactone Acid (V)*—200 mg. of lactone acetate (II) were saponified in 10 ml. of 2 per cent sodium hydroxide. The solution was cooled to 15° and acidified to pH 5 with vigorous stirring. The gummy, brown precipitate was triturated with aqueous acetone and the resulting gray solid (0.12 gm.) was crystallized several times from ethyl acetate-benzene, forming 0.03 gm. of nearly white needles, melting with effervescence at 135° in a preheated bath.

$C_{18}H_{20}O_4$ . Calculated, C 71.98, H 6.71; found, C 72.1, H 6.59

The acid is not stable, becoming colored after a few weeks storage.

*Improved Method for Preparation of Estrololactone Acetate*—A mixture of 15.6 gm. of estrone acetate and 0.18 gm. of *p*-toluenesulfonic acid in 180 ml. of a solution of peracetic acid (0.001 mole per ml.) in glacial acetic acid was allowed to stand in the dark for about 100 hours at 10°. The diluted reaction mixture yielded 13.8 gm. of crude crystalline estrololactone acetate, m.p. 144–149°. In several such experiments the yield was 80 to 85 per cent.

## SUMMARY

The oxidation of equilenin acetate with peracetic acid to the lactone acetate of bisdehydroestrolac acid is described. The chemical properties of the lactone show that it is similar to the oxidation products of other 17-keto steroids reported previously. The reaction temperature has been demonstrated to have a marked effect on the yield of lactonic oxidation products formed from 17-keto steroids with peracetic acid.

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# SOME IODINATED DERIVATIVES OF CHOLANIC ACID\*

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AND LOUISE ROMANOFF

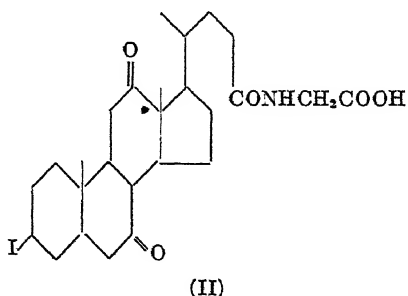
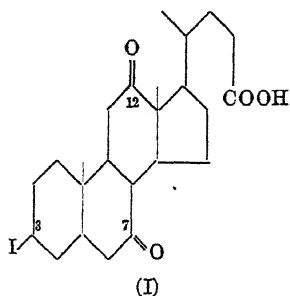
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(Received for publication, May 19, 1947)

This paper describes an attempt to prepare a radiopaque substance which would be concentrated in the biliary tract and serve as a diagnostic agent in the x-ray visualization of the gallbladder.

The compounds selected for synthesis were iodinated derivatives of cholanic acid and its glycine conjugate, glycocholic acid. It was felt that the similarity between these compounds and the naturally occurring bile acids might cause the iodinated derivatives to be concentrated in the gallbladder, thus rendering a visualization of this organ possible. However, a series of x-ray pictures and iodine determinations has shown that these compounds pass through the gastrointestinal tract virtually unabsorbed.

Treatment of methyl cholate with *p*-toluenesulfonyl chloride in pyridine solution gave the 3-*p*-toluenesulfonate as previously reported by Barnett and Reichstein (1). This product was oxidized in acetic acid with chromic oxide to the 3-*p*-toluenesulfonate of methyl 3-hydroxy-7,12-diketocholanic acid. Iodination with sodium iodide, followed by saponification and acidification, yielded 3-iodo-7,12-diketocholanic acid (I). The acid chloride of I, treated with a solution of glycine in sodium hydroxide according to the procedure of Cortese and Bauman (2), gave 3-iodo-7,12-diketoglycocholic acid (II).



\* The work described in this paper was supported by a grant from G. D. Searle and Company.

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The position of the iodine atom in these compounds was confirmed by the catalytic hydrogenation of I to 7,12-diketocholanic acid.

#### EXPERIMENTAL

All melting points were determined with total immersion of the thermometer stem. The microanalyses were performed under the direction of Dr. Robert T. Dillon, and the pharmacological tests by Dr. W. E. Hambourger, both of G. D. Searle and Company.

*3-p-Toluenesulfonate of Methyl Cholate*—A solution of 78 gm. of dried methyl cholate (m.p. 153.5–155.5°) in 110 ml. of pyridine was chilled to 10°, and a solution of 39 gm. of *p*-toluenesulfonyl chloride (10 per cent excess) in 75 ml. of pyridine was added dropwise over a period of 30 minutes. During this addition the temperature was maintained at 8–12°. The reaction mixture was then protected with a calcium chloride tube and allowed to stand at room temperature for 20 hours. A small amount of ice was added to decompose unchanged acid chloride and the mixture was diluted with 350 ml. of glacial acetic acid. The resulting solution was slowly poured into 3 liters of well stirred ice water containing 220 ml. of concentrated hydrochloric acid. The amorphous, white precipitate (105 gm.) was collected and, after thorough washing, was used in the next reaction without further purification.

*3-p-Toluenesulfonate of Methyl 3-Hydroxy-7,12-diketocholamate*—A solution of 105 gm. of the tosylated ester in 650 ml. of glacial acetic acid was chilled to 10°, and a solution of 34.5 gm. (20 per cent excess) of 85 per cent chromic oxide in 300 ml. of 50 per cent acetic acid was added dropwise over a period of 45 minutes, the temperature being maintained below 15°. The reaction mixture was allowed to stand at room temperature for 15 hours, and it was then added very slowly to 4 volumes of well stirred, cold water. The product was removed by filtration and washed well with sodium bicarbonate solution and water. Three recrystallizations from methanol provided 48 gm. (46 per cent) of a white, crystalline solid, m.p. 135–140°, which was used in the next reaction. The analytical sample melted at 142.5–143.5°.

$C_{32}H_{44}O_7S$ .	Calculated.	C 67.10, H 7.74, S 5.60
	Found.	" 67.07, " 7.80, " 5.77
		" 66.93, " 7.94, " 5.97

*Methyl 3-Iodo-7,12-diketocholamate*—A mixture of 45 gm. of the tosylated diketo ester, 45 gm. of sodium iodide, 450 ml. of acetonyl acetone, and a few small crystals of sodium thiosulfate was heated for 3 hours on the steam bath. The mixture was then cooled and added slowly to 2.5 liters

of water containing 30 gm. of sodium chloride. The pale yellow precipitate (42 gm.) was collected, washed, and used in the next reaction without purification. The analytical sample, recrystallized several times from methanol, melted at 171.5–172°.

$C_{25}H_{37}O_4I$ .	Calculated.	C 56.81, H 7.06
	Found.	" 56.92, " 7.32
		" 56.71, " 7.26

*3-Iodo-7,12-diketocholanic Acid (I)*—The crude iododiketo ester (40 gm.) was saponified in 680 ml. of methanol containing 8 gm. of potassium hydroxide. The methanol was removed by distillation of the diluted reaction mixture, and the product was precipitated with acid and recrystallized from aqueous methanol. The yield was 12 gm. (31 per cent) of product, melting at 225° or higher, in addition to a larger amount of impure material which could not be readily crystallized. The analytical sample melted at 229–232°, with decomposition.

$C_{24}H_{31}O_4I$ .	Calculated.	C 56.03, H 6.86, I 24.67
	Found.	" 56.05, " 6.96, " 24.69
		" 56.35, " 7.08

*3-Iodo-7,12-diketoglycocholanolic Acid (II)*—17 gm. of I was dried for 5 hours at 110° and dissolved in 150 ml. of redistilled thionyl chloride. This solution was allowed to stand at room temperature for 3 hours, during which time a continuous, slow stream of dry nitrogen was bubbled through it. The thionyl chloride was removed under diminished pressure and the residual cake was ground up in the reaction vessel with several portions of petroleum ether, which were removed under vacuum. These washings were continued until no acidic fumes remained. The solid product was then powdered and suspended in a solution of 37.5 gm. of glycine in 600 ml. of N sodium hydroxide. This suspension was stirred at room temperature for 3 hours, allowed to stand at room temperature for 20 hours, and finally stirred at 60° for an additional 3 hour period. It was then chilled and acidified to Congo red (with constant stirring) with hydrochloric acid. After it had stood in the refrigerator for several hours, the suspension was filtered and the product was washed well with water. The sparingly soluble glycine conjugate was obtained in crystalline form by the continuous extraction of this material with acetone in a Soxhlet apparatus, giving 8.2 gm. (43 per cent) of white crystals melting at 197.5–199°, with decomposition.

$C_{26}H_{38}O_5NI$ .	Calculated.	C 54.64, H 6.70
	Found.	" 54.85, " 6.68
		" 54.55, " 6.44

*Conversion of I to 7,12-Diketocholanic Acid*—A solution of 0.5 gm. of I in 60 ml. of 50 per cent aqueous methanol containing 0.4 gm. of sodium hydroxide and 0.2 gm. of 10 per cent palladinized norit catalyst was shaken with hydrogen for 30 minutes. After removal of the catalyst and the methanol, the product was precipitated with hydrochloric acid. Two recrystallizations from benzene-neohexane provided a white, crystalline product melting at 175.7–177°. When the substance was mixed with an authentic sample of 7,12-diketocholanic acid prepared from dehydrocholic acid according to the method of Borsche (3), the melting point was not changed.

#### SUMMARY

Four new derivatives of cholanic acid have been prepared, three of which are iodinated in the C<sub>3</sub> position.

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# THE DETERMINATION OF NITROGEN BALANCE INDEXES OF PROTEIN HYDROLYSATES IN DOGS\*

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*(Received for publication, July 25, 1947)*

Data obtained while feeding proteins or protein hydrolysates orally to dogs have demonstrated that the relationship between absorbed nitrogen ( $AN$ ) and urinary nitrogen ( $UN$ ) is described by the following equation

$$UN = (1 - K) (AN) + UN_0 \quad (1)$$

where  $UN_0$  is the excretion of urinary nitrogen on a protein-free diet and  $K$  is the nitrogen balance index of the dietary nitrogen (1). This is an equation for a straight line in regions of negative and low positive nitrogen balance where this balance is linearly related to absorbed nitrogen. Thus the index ( $K$ ), which is the rate of change of nitrogen balance with respect to absorbed nitrogen, is constant for any one protein in the region of negative balance. The index increases above normal, however, in hypo-proteinemic dogs, animals with reduced protein stores. The data of Frost and Risser (2) demonstrate that a linear relationship exists also between nitrogen balance and absorbed nitrogen in dogs fed a fibrin hydrolysate intravenously. There is need to expand these studies to investigate the application of Equation (1) to data obtained in animals fed hydrolysates intravenously. Experiments were planned, therefore, to determine under various experimental conditions the nitrogen balance indexes of different protein hydrolysates fed intravenously to normal and protein-depleted dogs.

## *Methods*

Normal parasite-free adult dogs were used in these experiments. The composition of the diet was described previously (3). The animals received 70 calories per kilo of body weight per day based upon the probable weight of the dogs as calculated from Cowgill's data (4).

\* The subject matter of this paper has been undertaken in cooperation with the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces. The opinions or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or endorsement of the War Department. These studies have also been supported in part by the Protein Metabolism Fund of the Bureau of Biological Research, Rutgers University. Some of these data were presented at the meetings of the Federation of American Societies for Experimental Biology, Chicago, May, 1947.

The protein hydrolysates were fed intravenously by means of a gravity drip apparatus. Infusions were conducted 18 hours after the last oral feeding. The dogs received oral diets which made up the remainder of the caloric intake on infusion days 1 hour after the end of the infusion.

The analytical methods used for the total urinary nitrogen,  $\alpha$ -amino nitrogen, ammonia, and urea have been reported previously (5). The "bound"  $\alpha$ -amino nitrogen was calculated by subtracting the free amino acid  $\alpha$ -amino nitrogen from the total  $\alpha$ -amino nitrogen determined after the urine had been hydrolyzed with an equal part of 12 N HCl for 6 hours at 120° and 15 pounds pressure.

### Results

Previous data have demonstrated that  $UN_0$  in Equation (1) varies with the physiological state of the dog, decreasing in value, for example, as the protein stores of the animal decrease (6). When  $K$  and  $AN$  are constant, Equation (1) becomes

$$UN = UN_0 + C \quad (2)$$

which describes a straight line with a slope of unity and a  $Y$  intercept of  $C$ .

The data plotted in Fig. 1, *A* illustrate the application of Equation (2) to some data obtained while feeding casein and an enzymatic casein hydrolysate to dogs. 100 mg. of nitrogen per kilo of body weight were absorbed each day. The slope of the straight line is unity. The intercept of this line ( $C$ ) is 20. Therefore

$$(1 - K)100 = 20 \text{ and } K = 0.80 \quad (3)$$

a value previously reported but calculated in a different way for casein and its hydrolysate (1).

The line in Fig. 1, *A* becomes curvilinear when  $UN_0$  decreases below 60 mg. per day per kilo of body weight. These low values for  $UN_0$  were obtained on dogs depleted in protein stores. Under these conditions the value of  $K$  increases, which means that the utilization of nitrogen is greater in the depleted than in the normal dog. Allison, Seeley, Brown, and Anderson (6), for example, reported a nitrogen balance index of 0.96 for casein and casein hydrolysate in the protein-depleted dog.

The data illustrated by the circles in Fig. 1, *B* were obtained while feeding a protein-free diet for 3 days followed by a 1 day intravenous feeding (120 mg. of nitrogen per kilo of body weight) of an acid hydrolysate of crude fibrin, at the rate of 1 mg. of N per minute per kilo of body weight. This hydrolysate was fed 18 hours after the last protein-free meal. The daily excretion of urinary nitrogen while the dogs were receiving the protein-free diet ( $UN_0$ ) and the hydrolysate ( $UN$ ) are plotted in Fig. 1, *B*.

The data recorded with the squares were obtained in exactly the same manner as those represented by the circles except that  $UN_0$  was determined by feeding a diet containing whole egg for 3 days instead of the protein-free diet. During this period, each dog received 90 mg. of whole egg nitrogen

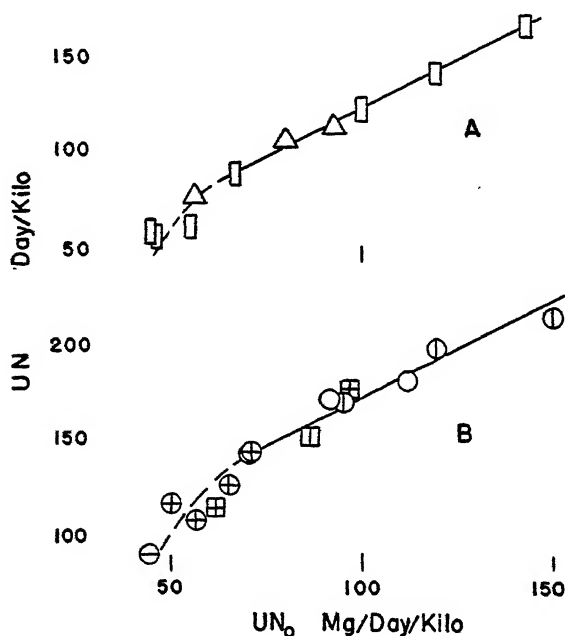


FIG. 1, A. Urinary nitrogen ( $UN$ ) (mg. per day per kilo of body weight) in dogs fed orally casein nitrogen ( $\square$ ) or casein hydrolysate nitrogen ( $\Delta$ ) plotted against urinary nitrogen ( $UN_0$ ) in the same dogs fed a protein-free diet. Each dog received 100 mg. of nitrogen per day per kilo during the nitrogen feeding period.

FIG. 1, B. Urinary nitrogen ( $UN$ ) in dogs fed a fibrin hydrolysate intravenously plotted against urinary nitrogen ( $UN_0$ ) in the same dogs fed a protein-free diet. The hydrolysate was fed at the rate of 1 mg. of N per minute per kilo and in an amount equal to 120 mg. of N per day per kilo.  $\square$  represents data in which  $UN_0$  was obtained while feeding whole egg instead of the protein-free diet.  $\circ$  represents data obtained while feeding a protein-free diet, followed by the feeding of an acid hydrolysate of crude fibrin. The marks in the circles and squares identify different dogs.

per kilo of body weight daily. The nitrogen balance index of whole egg being unity,  $UN_0$  was not altered by the addition of the egg protein. Egg protein diet was used, therefore, to determine  $UN_0$ , preventing the gradual depletion in nitrogen which occurs during periods of nitrogen-free feeding.

The slope of the linear portion of the curve in Fig. 1,  $B$  is unity and the  $Y$  intercept is 72; therefore,

$$(1 - K)120 = 72 \text{ and } K = 0.40 \quad (4)$$

The line becomes curvilinear at values of  $UN_0$  less than 70 mg. per day per kilo. These low values for  $UN_0$  were obtained on one dog and are values which are associated with increased nitrogen balance indexes, indicative of an increased utilization of nitrogen.

TABLE I

*Rate of Infusion of Fibrin Hydrolysate, Urinary Nitrogen (UN), Urinary Nitrogen on a Protein-Free Diet (UN<sub>0</sub>), and Nitrogen Balance Index (K)\**

Dog No.	Rate of infusion	Urinary nitrogen, <i>UN</i>	Protein-free urinary nitrogen, <i>UN</i> <sub>0</sub>	Nitrogen balance index, <i>K</i>
	<i>mg. N per min. per kg.</i>	<i>mg. per day per kg.</i>	<i>mg. per day per kg.</i>	
57	0.5	173	105	0.43
	0.5†	161	88	0.39
	1.0	193	115	0.35
	1.0‡	171	92	0.34
	7.0	172	100	0.40
65	7.0†	162	81	0.33
	1.0	124	66	0.52
	1.0†	88	44	0.64
	1.0	108	57	0.57
	1.0†	119	50	0.43
	1.0‡	103	64	0.68
	5.5	151	85	0.45
	5.5†	119	64	0.54

\* Calculated from the equation  $UN = (1 - K)(AN) + UN_0$ , where the nitrogen intake ( $AN$ ) was 120 mg. of N per day per kilo of body weight.

† 5 per cent glucose added to hydrolysate.

‡ 10 per cent glucose added to hydrolysate.

Table I is a record of data obtained during a study of the effects of the rate of infusion and of the addition of glucose on the nitrogen balance index of an acid hydrolysate of crude fibrin. The indexes were calculated from Equation (1). Each index represents a separate experiment consisting of a 3 day protein-free period followed by 1 day of intravenous feeding. One dog (No. 57) was selected to represent the normal, the other (Dog 65) the abnormally low  $UN_0$ . These data demonstrate that a change in rate of flow from 0.5 to 7.0 mg. of N per minute per kilo of body weight has no significant effect on the nitrogen balance index. Neither does the addition of glucose to the fibrin hydrolysate alter the index significantly. The average index for Dog 65 with the low value

for  $UN_0$  is 0.53, which is greater than the average index of 0.38 obtained for the normal dog, No. 57.

Table II summarizes experiments on three dogs to determine the effects of the daily feeding of fibrin hydrolysate nitrogen on the nitrogen balance indexes. Two dogs (Nos. 44 and 63) with normal values for  $UN_0$  and one

TABLE II

*Nitrogen Source, Days on Experiment, Nitrogen Intake (AN), Urinary Nitrogen (UN), and Nitrogen Balance Index (K)*

$K$  is calculated from the equation,  $UN = (1 - K)(AN) + UN_0$ . The nitrogen intake (AN) was obtained by feeding the hydrolysate intravenously at the rate of 1 mg. of N per minute per kilo of body weight. The  $UN$  for whole egg was used for  $UN_0$ .

Nitrogen source	Day	Absorbed nitrogen	Dog 44		Dog 63		Dog 65	
			UN	K	UN	K	UN	K
			mg. per day per kg.	mg. per day per kg.	mg. per day per kg.	mg. per day per kg.	mg. per day per kg.	mg. per day per kg.
Whole egg	0-3	90	86		96		64	
Crude fibrin hydro- lysate	4	120	155	0.43	177	0.33	113	0.60
Crude fibrin hydro- lysate	5	120	167	0.33	172	0.37	117	0.56
Crude fibrin hydro- lysate	6	120	155	0.40	164	0.33	120	0.60
Crude fibrin hydro- lysate	7	120	161	0.35	160	0.36	120	0.60
Whole egg	8-10	90	83		83		71	
Fibrin hydrolysate	11	120			161	0.35	130	0.51
Dog 73								
Whole egg	0-3	90	96		83		128	
Casein hydrolysate	4-5	120	133	0.69	124	0.66	163	0.71
" "	5-6	120	122	0.63	118	0.63	151	0.67
Whole egg	7-9	90	78		73		112	
Dog 74								
" "	10-16	120	78		78		109	
Casein hydrolysate	17-18	120	117	0.67	126	0.60	151	0.65
" "	19-20	120	101	0.67	111	0.60	122	0.68
Whole egg	21-24	120	61		63		84	

dog (No. 65) with an abnormally low  $UN_0$  were chosen for this study. The dogs were fed the whole egg protein (90 mg. of N per day per kilo of body weight) for 3 days to determine the  $UN_0$ . They were transferred to the protein-free diet during the days of intravenous feeding. The nitrogen balance indexes were calculated according to Equation (1) from values for  $UN_0$  nearest to the days of intravenous feeding. The indexes for Days



4 and 5 were calculated, for example, with the  $UN_0$  obtained during Days 0 to 3, while the indexes for Days 6 and 7 were calculated with the  $UN_0$  obtained during Days 8 to 10.

The average nitrogen balance index for the fibrin hydrolysate obtained by feeding the two normal dogs is 0.37, close to the average value of 0.4 calculated from the curve in Fig. 1, *B*. The average index obtained in Dog 65 with the low  $UN_0$  is 0.57, which compares favorably with the average value of 0.54 from data on this same dog in the experiments recorded in Table I.

Table II records another series of experiments in which the nitrogen balance indexes of an acid hydrolysate of casein were determined over a period of 3 weeks in different normal dogs. In these experiments average indexes over periods of 2 days were calculated instead of daily indexes. The value for  $UN_0$  decreased during these experiments, even though the whole egg protein nitrogen was increased to 120 mg. per day per kilo of body weight. This decrease in  $UN_0$  is undoubtedly the result of loss of protein stores during intravenous feeding when the dogs were in negative nitrogen balance. Changes in  $UN_0$  are, however, all within the normal range and do not alter the value of  $K$  significantly. There is good agreement in the values for the nitrogen balance index of the casein hydrolysate determined in the various dogs.

The data illustrated in Fig. 2 demonstrate that the rate of infusion affects the concentration of amino nitrogen in the plasma and the excretion of amino compounds in the urine. The data in Fig. 2, *A* were obtained by analyzing plasma samples taken immediately after the end of the infusion of the crude fibrin hydrolysate. These analyses prove that the amino nitrogen concentration increases in a curvilinear manner as the rate of infusion increases from 0 to 7 mg. of N per minute per kilo of body weight. Vomiting and nausea occurred when the hydrolysate was given at rates greater than 3 mg. of N per minute per kilo when the plasma amino nitrogen increased to more than 15 mg. per cent. The data in Fig. 2, *B* demonstrate that the excretion of the amino nitrogen increases as the rate of infusion increases. No generalization can be made concerning the effect of rate of infusion on the retention of nitrogen from all hydrolysates, since they present different patterns of amino acids to the animal. It is interesting to note, however, that the excretion of urinary amino nitrogen approaches a limit, the slope of the curve decreasing rapidly at an infusion rate of approximately 5 mg. of nitrogen per minute per kilo. Similarly, Silber, Seeler, and Howe (7) found that there was no increase in amino acid excretion when the infusion of a mixture of amino acids was increased from 6 to 12 mg. of N per minute per kilo.

The rate of excretion of  $\alpha$ -amino, ammonia, and urea nitrogen and other

forms of nitrogen during the day of infusion of the partial hydrolysate of crude fibrin is illustrated in Fig. 3. The 1st hour in Fig. 3 represents the rate of excretion of the various forms of nitrogen before the infusion was started. The infusion was given during the 1st to 3rd hours. Fig. 3 illustrates the marked rise above control values in the rate of excretion of  $\alpha$ -amino nitrogen and of urea nitrogen during and after infusion. The rate of excretion of  $\alpha$ -amino nitrogen fell rapidly toward control values within an

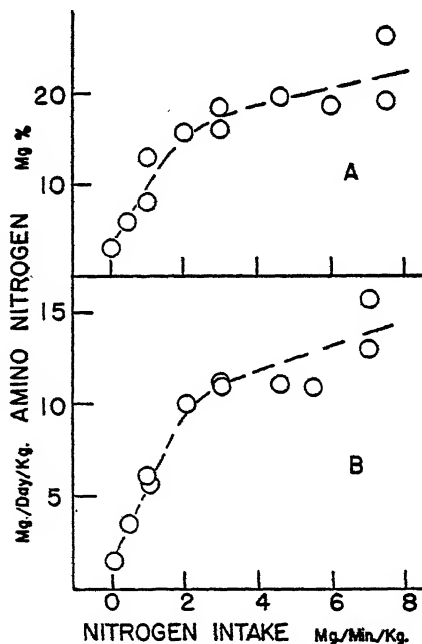


FIG. 2, A. Plasma  $\alpha$ -amino nitrogen at the end of the intravenous infusion of a fibrin hydrolysate plotted against the rate of infusion of nitrogen.

FIG. 2, B. Urinary  $\alpha$ -amino nitrogen in the same experiments illustrated in Fig. 2, A.

hour after the infusion ended. The rate of excretion of urea nitrogen continued to rise after the infusion, returning to control values several hours thereafter. The excretion of ammonia nitrogen was not altered by the infusion, but the excretion of other forms of nitrogen increased during and for several hours after the infusion, a result primarily of a rise in the excretion of "bound"  $\alpha$ -amino nitrogen.

The intake and output of  $\alpha$ -amino nitrogen, both free and bound, are illustrated by the data recorded in Table III. Measurements of these forms of nitrogen represent preliminary experiments to determine the utilization

of polypeptides, as well as of free amino acid  $\alpha$ -amino nitrogen. It is interesting to note that amino nitrogen can be partitioned differently between free and bound forms in these hydrolysates and yet have the same utilization as measured by nitrogen balance indexes.

The acid hydrolysate, aminosol, prepared from fibrin (2) has a higher nitrogen balance index than the partial hydrolysate made from crude fibrin.

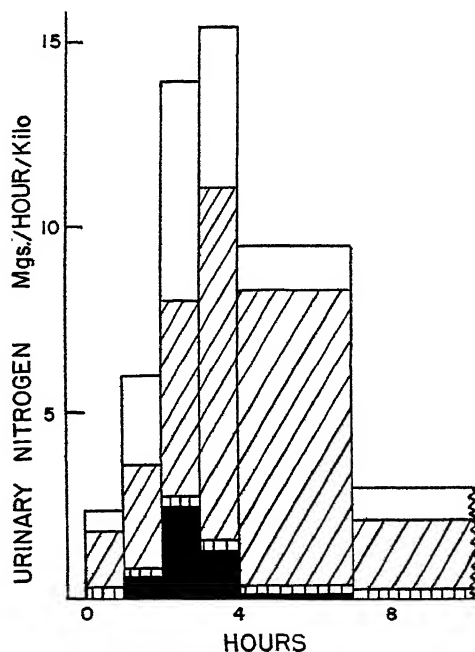


FIG. 3. The rate of urinary nitrogen excretion plotted against time in hours. The data for the 1st hour represent the control. 120 mg. of fibrin hydrolysate nitrogen per kilo of body weight were infused during the 1st to 3rd hours. The black blocks represent the rate of excretion of  $\alpha$ -amino nitrogen, the blocks with vertical lines the rate of excretion of ammonia nitrogen, the blocks with slanted lines the rate of excretion of urea nitrogen, and the white blocks the rate of excretion of other forms of nitrogen.

The difference between these hydrolysates may be due in part to the degrees of hydrolysis, but also is probably associated with the fact that the crude fibrin was contaminated with other plasma proteins of lower nutritive value. Frost and Risser (2) have reported a nitrogen balance index of 0.83 to 0.86 for a fibrin hydrolysate corresponding to aminosol. These higher indexes were obtained in dogs depleted in proteins, animals which yield higher than normal indexes. The data in Table IV demonstrate the increase above normal in the nitrogen balance index of supprotein when fed intravenously to protein-depleted dogs.

TABLE III

*Hydrolysate, Amino Nitrogen Infused (Free and Bound), Urinary Amino Nitrogen Excreted (Free and Bound) during Protein-Free Feeding and Infusion Periods, and Nitrogen Balance Indexes of Hydrolysates*

Average data obtained on three dogs while being fed intravenously for 4 days according to the methods described in the text.

Hydrolysate*	Amino nitrogen infused		Urinary amino nitrogen				Nitrogen balance index
	Free	Bound	Control		Infusion†		
			Free	Bound	Free	Bound	
	mg. per day per kg.	mg. per day per kg.	mg. per day per kg.	mg. per day per kg.	mg. per day per kg.	mg. per day per kg.	
Fibrin (crude).....	33	57	1.7	3.6	6.2	17.9	0.36
Aminosol.....	60	32	1.9	3.1	6.0	9.8	0.66
Supprotein.....	73	14	1.5	3.0	3.4	4.4	0.67
Paranamine. 6 %.....							0.66

\* The authors thank the Abbott Laboratories for the aminosol, Eli Lilly and Company for the supproteine, and Frederick Stearns and Company for the paranamine. We thank Dr. D. V. Frost for preparing the acid hydrolysate of crude fibrin.

† 120 mg. of hydrolysate nitrogen per day per kilo of body weight were infused at the rate of 1 mg. of N per minute per kilo of body weight.

TABLE IV

*Effect of Depletion in Proteins (Reflected by Low Value for  $UN_0$ ) on Nitrogen Balance Index*

These are average data obtained while feeding 120 mg. of supproteine nitrogen per day per kilo of body weight intravenously to three normal and two protein-depleted dogs at the rate of 1 mg. of N per minute per day.

Normal			Protein-depleted		
UN	UN <sub>0</sub>	K	UN	UN <sub>0</sub>	K
mg. per day per kg.	mg. per day per kg.		mg. per day per kg.	mg. per day per kg.	
120	80	0.67	69	47	0.82

TABLE V

*Nitrogen Balance Index of Supproteine Fed Orally to Adult Dogs*

Dog No.	Absorbed nitrogen	Urinary nitrogen	Protein-free urinary nitrogen	Nitrogen balance index
	mg. per day per kg.	mg. per day per kg.	mg. per day per kg.	
31	1.81	1.95	1.57	0.79
	2.43	2.10	1.62	0.80
44	1.95	1.64	1.26	0.81
63	1.78	1.40	1.24	0.91
	2.52	1.89	1.43	0.82
Average.....				0.82

All of the hydrolysates listed in Table III have a higher index when fed orally than when fed intravenously. The order of difference is illustrated by the data on supprotein recorded in Table V, in which the average nitrogen balance index is 0.82, considerably higher than the 0.67 for intravenous feeding. The nitrogen balance index of crude fibrin hydrolysate fed orally was determined to be 0.64, also much higher than the 0.4 characteristic of intravenous feeding. Entrance of the hydrolysate through the gastrointestinal tract can present a different pattern of amino acids to the animal than when introduced through the vein because of enzymatic digestion and absorptive processes in the gut, and of more direct action of liver tissue on the absorbed products, other tissues of the body such as the kidney not being affected as directly by oral as by intravenous feeding.

#### SUMMARY

1. The relationship between absorbed nitrogen ( $AN$ ) and urinary nitrogen ( $UN$ ) is described by the equation,  $UN = (1-K) (AN) + UN_0$ , where  $UN_0$  is the excretion of urinary nitrogen on a protein-free diet and  $K$  is the nitrogen balance index of the hydrolysate.

2.  $UN_0$  is a function of the protein stores, decreasing in value as the stores decrease.  $K$  is a constant in the region of negative nitrogen balance, provided  $UN_0$  is greater than 70 mg. of N per day per kilo of body weight. When  $UN_0$  is less than this, the nitrogen balance index is variable, increasing as  $UN_0$  decreases. Thus high indexes are obtained in protein-depleted dogs.

3. The rate of infusion and the presence or absence of glucose in the hydrolysate did not affect the nitrogen balance index of a crude fibrin hydrolysate.

4. The rate of excretion of  $\alpha$ -amino nitrogen, both "bound" and free, and of urea nitrogen increased above control values during and after infusion. The excretion of ammonia nitrogen was not altered by the infusion.

5. The nitrogen balance indexes were higher in orally than in intravenously fed hydrolysates.

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# THE METABOLISM OF ISOTOPIC LACTIC ACID AND ALANINE IN THE PHLORHIZINIZED ANIMAL

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Although there is ample evidence that lactic acid and alanine are glyco-genic, the availability of isotope techniques has raised a number of questions concerning the fate of these substances in both the normal and experimentally "diabetic" animal. An earlier report (1) upon the administration of carboxyl-labeled alanine to phlorhizinized rats indicated that relatively little of the fed carbon was recovered in the urinary glucose. Conant and coworkers (2) as well as Vennesland *et al.* (3) have similarly demonstrated that administration of radioactive lactate to previously fasted rats leads to the deposition of liver glycogen containing but a small portion of the administered radioactivity. Stetten and Boxer (4) have supplied evidence indicating that the conversion of glyco-genic substances to glycogen may be a relatively minor process and that there is in many instances a major diversion of such substances into fat.

This report is concerned with a further study of the ultimate disposition of the carbon atoms of administered lactate in the fasted phlorhizinized rat (5). More extensive data upon the conversion of isotopic alanine to glucose in similar animals are also presented.

## EXPERIMENTAL

*Syntheses*—Isotopic DL-alanine containing  $C^{13}$  in the carboxyl position was prepared from 0.44 gm. of acetaldehyde and 0.5 gm. of isotopic sodium cyanide<sup>1</sup> by the Strecher synthesis as modified by Kendall and McKenzie (6). With these amounts, a 48 per cent yield of alanine was obtained. N 15.60; theory 15.71. The product contained 9.41 atoms per cent excess  $C^{13}$ .

Lactic acid containing  $C^{14}$  in the  $\alpha$  or  $\beta$  position was prepared from  $BaC^{14}O_3$ , as described by Sakami, Evans, and Gurin (7).

*Animal Experiments*—Fasting rats were treated with subcutaneous injections of phlorhizin in oil, 120 to 200 mg. of phlorhizin per kilo per day being employed. The G:N ratios on the 3rd day ranged from 2.22 to 3.51 and on the 4th day from 2.42 to 3.81 in eleven animals.

In Experiment 1, 200 mg. of carboxyl-labeled alanine in water were

<sup>1</sup> We wish to thank Dr. Harold C. Urey for supplying us with this material.

injected subcutaneously at the beginning of the 3rd day and the urine collected for 8 hours and for the following 16 hours respectively. Glucose was separately isolated from both urines.

In Experiment 2, another sample of alanine containing less  $C^{13}$  (1.74 atoms per cent excess) was employed. 100 mg. of alanine in 2 ml. of water were injected at the beginning of the 2nd day. 4 hours later another injection of an equal quantity of isotopic alanine was given. Urine was collected for 8 hours after the first injection and subsequently for the following 16 hours.

For Experiment 3, radioactive sodium lactate containing  $C^{14}$  in the  $\alpha$  or  $\beta$  positions was employed. On the 3rd day of fasting and phlorhizin treatment, a total of 500 mg. of sodium lactate was administered by stomach tube in five equal doses given every 30 minutes. Urine was collected during the first 6 hours of the experiment and subsequently for another 18 hours. Respiratory carbon dioxide was collected during the first 4 hours. Glucose was isolated from the urines and the carbon of the ketone bodies obtained by means of Denigès' reagent, as described below.

#### *Isolation Procedures*

*Glucose*—For the isolation of glucose, the various samples of urine were first clarified by the procedures of Neuberg and Kerb (8) and West, Scharles, and Peterson (9) by treating with 10 per cent  $HgSO_4$  in 5 per cent  $H_2SO_4$  and subsequently shaking with finely powdered  $BaCO_3$  until the suspension gave a faintly alkaline reaction. 5 volumes of alcohol were then added and the suspension chilled for several hours. After filtration and suitable washing of the precipitate, the combined fluid was evaporated *in vacuo* to dryness. The residue was extracted with three 5 ml. portions of hot methanol and the extract concentrated *in vacuo* to dryness. The glassy residue was dissolved in a small volume of glacial acetic acid and treated with *p*-nitrophenylhydrazine in acetic acid. After standing at room temperature for several days, the crystalline *p*-nitrophenylhydrazone was collected, washed with minimal cold ethanol to remove acetic acid, and the compound recrystallized from boiling 95 per cent ethanol. Usually one recrystallization yielded material which had the correct melting point and gave no depression upon admixture with an authentic sample of glucose *p*-nitrophenylhydrazone. If the nitrogen analysis was also satisfactory (the nitrogen analyses ranged from 13.26 to 13.50; theory 13.33), glucose was recovered from the hydrazone by refluxing in minimal 50 per cent ethanol for 3 to 4 hours with a slight excess of benzaldehyde. After addition of water, the precipitate was removed, washed with water, and the aqueous fraction extracted several times with ether and subsequently with ether containing a little butyl

alcohol. The colorless aqueous extract was concentrated *in vacuo* to a syrup and crystallized by the addition of warm ethanol.

*Ketone Bodies*—1 ml. aliquots of the urines obtained in Experiment 3 were treated with  $\text{CuSO}_4$  and lime according to the Van Slyke procedure for the estimation of ketone bodies (10). The filtrates were acidified to pH 6 with dilute HCl and aerated with  $\text{CO}_2$ -free air. Denigès' reagent and 1 ml. of 50 per cent  $\text{H}_2\text{SO}_4$  were added and the solution heated under reflux for 1 hour. The evolved  $\text{CO}_2$  representing the carboxyl carbon of acetoacetate was trapped in barium hydroxide solution. The precipitated mercury-acetone complex was collected by centrifugation and washed. The supernatant was again heated under reflux and treated with dichromate for the oxidation of  $\beta$ -hydroxybutyrate to acetoacetate. The  $\text{CO}_2$  representing the carboxyl carbon of  $\beta$ -hydroxybutyrate was trapped in barium hydroxide and the mercury-acetone precipitate collected separately and washed. The mercury-acetone fractions were dissolved in 1 N HCl and the acetone distilled into Denigès' reagent. These precipitates were collected, washed, and analyzed for radioactivity.

A control experiment was performed in order to eliminate any possibility that small amounts of radioactive lactate excreted in the urine could have contaminated the mercury-acetone precipitates. 10 mg. of  $\alpha$ -,  $\beta$ -labeled radioactive sodium lactate with an activity of  $1.54 \times 10^6$  counts per minute per mole of carbon were mixed with 100 mg. of  $\beta$ -hydroxybutyrate and added to 10 ml. of urine. The urine was clarified as described above and heated under reflux with Denigès' reagent and dichromate. The mercury-acetone precipitate was collected and washed several times with water. The material upon analysis was found to contain a trace of radioactivity ( $1 \times 10^4$  counts per minute per mole of carbon). The recovery of  $\text{C}^{14}$  in the mercury-acetone fraction was less than 0.7 per cent. A dilution of this order of magnitude could not have been detected with the weakly radioactive lactate employed in these experiments. It is of interest to note that even this trace of radioactive contaminant can be removed by dissolving the mercury-acetone precipitate in 1 N HCl followed by distillation of the acetone into fresh Denigès' reagent. The resulting product was found to contain no detectable amount of radioactivity.

*Fat*—Liver and muscle were separately ground with 5 per cent trichloroacetic acid. After filtration the precipitates were refluxed with boiling acetone for 5 hours to remove fat. Crude fat samples were obtained by evaporation of the acetone.

*Liver and Muscle Glycogen*—In Experiments 1 and 2, the liver and muscle glycogen were isolated by extraction with trichloroacetic acid. Several extractions of the solution with ether to remove lipides were followed by precipitation of the glycogen with ethanol.



*Radioactivity Measurements*—All of the measurements were made with a bell-shaped thin mica window Geiger counter (Technical Associates), upon samples of  $\text{BaCO}_3$  obtained by wet oxidation (11) of organic substances. The layer of  $\text{BaCO}_3$  was prepared as follows: the precipitate, after thorough washing and drying, was ground under acetone and the suspension rapidly poured on a raised metal disk contained in a glass cup. A thin wire loop placed under the disk is satisfactory for withdrawing it. With a fairly snug fit, not too much precipitate is lost around the sides of the disk. The acetone was evaporated by means of a lamp placed 4 to 5 inches above the cup. By this means a uniform layer of barium carbonate is obtained. The disk can then be lifted out of the cup by means of the wire loop.

With a standard preparation of uniform specific radioactivity, plates of varying thickness were prepared to determine the relationship of recorded activity in the counter to plate thickness. The relationship was found to be identical with that described by Reid (12). Samples containing 17 to 18 mg. of  $\text{BaCO}_3$  per sq. cm. were found to behave as infinitely thick layers. As a rule, plates were prepared containing 8 to 18 mg. of  $\text{BaCO}_3$  per sq. cm. and corrected to activity at infinite thickness. With background counts of  $10 \pm 2$  per minute, samples of unknowns were counted sufficiently long to give several hundred counts. From the corrected activity, counts per minute per mole of carbon were calculated.

### Results

In Table I are summarized the results obtained in Experiments 1 and 2. In Experiment 1, 200 mg. of alanine were injected subcutaneously in a single dose, whereas in Experiment 2 two injections of 100 mg. of alanine respectively were given 4 hours apart. Although the data are not complete enough for accurate estimations, a rough calculation of the extra sugar by a procedure similar to Lusk's indicates that approximately 60 to 70 per cent of the administered alanine was converted to glucose during the collection. Although three of the urinary glucose samples contained an excess of  $\text{C}^{13}$ , the total recovery of isotope in the glucose amounted to only 1 to 5 per cent. The excess of  $\text{C}^{13}$  in the muscle and liver glycogen was not significant.

In order to determine the position of the isotope, a sample of the isotopic glucose was generously degraded for us by Dr. Harland G. Wood, who found the isotope to be located preponderantly in positions 3 and 4 of the glucose molecule. These results, although preliminary, agree with the predictions of other investigators (13, 14).

In Table II are summarized the urinary nitrogen and glucose values obtained in Experiment 3. After administration of 500 mg. of  $\alpha$ -,  $\beta$ -labeled

sodium lactate an estimated 300 to 400 mg. of "extra glucose" were excreted in the urine, indicating good conversion of the lactate to glucose.

TABLE I  
*Administration of Carboxyl-Labeled Alanine*

	Experiment 1				Experiment 2		
	Day of fasting and phlorhizin						
	1	2	3 (a)*	3 (b)†	1	2 (a)‡	2 (b)
Urinary glucose, mg.	1190	976	221	470	758	328	291
“ N, mg.	235	336	58		190	120	121
G:N ratio	5.06	2.88	3.81		3.99	2.74	2.40
“Extra sugar,” mg.			144			116	
Conversion of alanine to “extra” glu- cose, %			70			60	
C <sup>13</sup> atom % excess							
Administered alanine			9.41			1.74	
Isolated glucose (8 hr. urine)			0.10			0.05	
“ “ (9-24 hr. urine)			0.04			0.00	
Muscle glycogen			0.02			0.00	
Liver “			0.02			0.00	
Recovery of isotope in urinary glucose, %			1.2			4.7	

(a), urine collected during first 8 hours; (b), urine collected from 9th to 24th hour.

\* 200 mg. of isotopic alanine were injected subcutaneously at the beginning of the period.

† This urine was contaminated with blood.

‡ 100 mg. of isotopic alanine injected subcutaneously at the beginning and 100 mg. after 4 hours.

TABLE II  
*Administration of Lactate Labeled with C<sup>14</sup>*

	Day of fasting and phlorhizin			
	1	2	3 (a)	3 (b)
Urinary glucose, mg.....		853	469	790
" N, mg.....		300	80	222
G:N ratio.....		2.84	5.86	3.56
Extra glucose, mg.....			242	160

(a), urine collected during first 6 hours; (b), urine collected from 7th to 24th hour.

Radioactivity measurements indicated that the glucose isolated from the urine excreted during the first 6 hours contained  $4.64 \times 10^4$  counts per

minute per mole of carbon (Table III), whereas the administered lactate contained  $26.4 \times 10^4$  counts. 20 per cent of the administered radioactivity was recovered in the glucose. The glucose isolated from the 6 to 24 hour urine was approximately one-fourth as radioactive. Respiratory carbon dioxide was collected over half-hour periods for 4 hours after administration of the lactate. The radioactivity varied from 2.16 to  $4.55 \times 10^4$  counts per minute per mole of carbon. Approximately 17 per cent of the isotopic carbon of lactic acid was completely oxidized to carbon dioxide and excreted in 4 hours. The somewhat higher radioactivity of the glucose indicates a direct conversion of lactate carbon to glucose in addition to incorporation of isotopic  $\text{CO}_2$  in glucose.

TABLE III  
*Recovery of Isotope after Administration of  $\alpha,\beta$ -Labeled Lactate*

Substance	Counts per min. per mole carbon $\times 10^4$	Total counts administered or recovered	Recovery  <i>per cent</i>
Administered lactate.....	26.4	3580	
Respiratory $\text{CO}_2$ (4 hrs.).....	2.16-4.55	620	17.3
Urinary glucose (6 hrs.).....	4.64	720	20.0
“ “ (7-24 hrs.).....	1.10	294	8.2
Ketone bodies (6 hrs.)		267	7.5
Carboxyl of acetoacetate.....	5.40		
Acetone “ “.....	5.04		
Carboxyl “ $\beta$ -hydroxybutyrate.....	5.70		
Acetone “ “.....	5.10		
Ketone bodies (7-24 hrs.)		97	2.7
Carboxyl fraction.....	2.88		
Acetone “.....	3.00		
Liver fat.....	1.80	260	7.3
Muscle “.....	0.96	800	22.4

The ketone bodies of the urine were slightly more radioactive than the glucose and markedly more so than the respiratory carbon dioxide. The acetoacetate and the  $\beta$ -hydroxybutyrate were equally radioactive. Inasmuch as the radioactivity was approximately the same in the carboxyl and acetone fractions, *it is clear that every carbon in the ketone bodies was labeled with  $\text{C}^{14}$ .*

The liver and muscle fat were radioactive but significantly less so than the urinary ketone bodies. The isotope recovered in the fat and urinary ketone bodies amounted to 40 per cent of the total administered.

The liver and muscle proteins were separately hydrolyzed and partitioned into dicarboxylic, dibasic, and monoamino acid fractions. The

radioactivity was either zero or insignificant in most of these preparations. A little radioactivity was detected in the phosphotungstate precipitate and was attributed to the incorporation of metabolic carbon dioxide into the guanido group of arginine.

#### DISCUSSION

It is apparent that in the phlorhizinized animal the metabolic pathways of alanine and lactic acid are divergent. Whereas only 1 to 5 per cent of the isotopic carbon of alanine was recovered in the urinary glucose, 25 to 30 per cent of the isotopic carbon of lactate was changed to glucose. The dilution of isotope in both instances was quite different. If one assumes that carboxyl-labeled alanine is converted to glucose containing isotope located preponderantly in the 3 or 4 position, then one may calculate the resulting dilution in Experiment 2 to be 1:17.

$$\frac{1.74 \times 3}{0.05 \times 6} = 17.4$$

In the case of lactate, the dilution is considerably smaller. Assuming that the resulting glucose is labeled either in the 1 or 2 position or in the 5 or 6 position, then the dilution is

$$\frac{26.4 \times 10^4 \times 3}{4.64 \times 10^4 \times 6} = 2.8$$

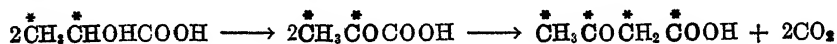
In neither case has a correction been made for the incorporation of metabolic  $\text{CO}_2$  into the glucose so that the dilution figures are minimal. If one assumes in Experiment 3 that position 3 or 4 of the glucose molecule is also radioactive and has a radioactivity equal to the maximal values obtained with respiratory  $\text{CO}_2$  ( $4.55 \times 10^4$ ), then the maximal dilution is 1:3.4.

$$\frac{26.4 \times 10^4 \times 3}{\left(4.64 - \frac{4.55}{6}\right) \times 10^4 \times 6} = 3.4$$

It appears reasonable to assume that a considerable portion of the administered alanine is promptly incorporated into tissue protein. Should this be the case, it follows that a relatively small proportion is oxidatively deaminized and mixed with the circulating carbohydrate pool. In any event it seems clear that, although injected alanine may cause the excretion of a large amount of "extra" glucose in the phlorhizinized animal, the administered carbons are not the ones excreted. Lactate on the other hand appears to be promptly converted to glucose. Under these experimental conditions, 20 per cent of the radioactive carbon of the administered lactate was recovered in the glucose isolated from the first 6 hour urine.

The dilution was less than that observed by Vennesland *et al.* (3) in the normal fasting animal.

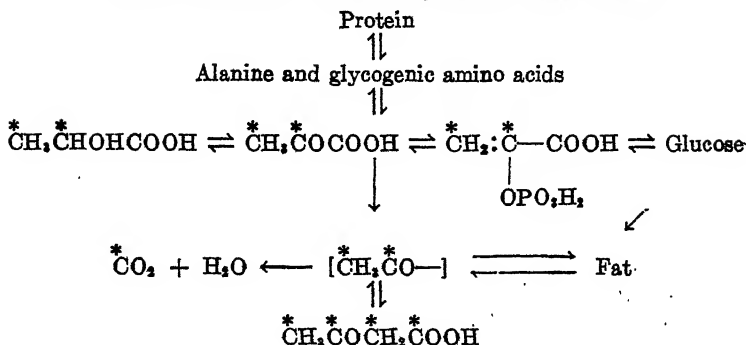
The conversion of lactate to ketone bodies is not surprising in view of the extensive literature indicating that liver slices (15, 16) and homogenates of liver (17) are capable of transforming pyruvate into acetoacetate. Lehninger (17) has demonstrated that washed homogenates of liver are capable of quantitatively transforming pyruvate into acetoacetate in the absence of the 4-carbon dicarboxylic acids (fumarate, succinate, etc.). The results obtained with the phlorhizinized animal indicate the following series of metabolic reactions.



The fact that uniform concentrations of isotope are found in all four possible positions of acetoacetate is strong evidence that pyruvate can be directly converted to ketone bodies without intermediate formation of fat. The observation that one finds significantly greater radioactivity in the carbon atoms of the ketone bodies than is present in the carbon of liver and muscle fat adds further support to this direct metabolic pathway. In this connection it is of interest that in a separate report (18) we have demonstrated that  $\alpha,\beta$ -labeled pyruvate is converted to completely labeled acetoacetate by washed liver homogenates.

The recovery of 30 per cent of the administered radioactivity in the liver and muscle fat confirms the observations of Stetten and Klein (19) that, in the phlorhizinized animal, glycogenic substances are to a very considerable extent metabolized via fat. Our recovery of isotope in the fat is minimal since we were unfortunately unable to analyze subcutaneous and depot fat. In any event it is clear that *approximately half of the isotope of the administered lactate was converted into fat and ketone bodies.*

There is ample evidence in the literature (20) which indicates that lactate is an antiketogenic substance. This fact must be reconciled with the present finding that  $\alpha,\beta$ -labeled lactate can contribute its carbon to all four carbons of acetoacetate and  $\beta$ -hydroxybutyrate. If "extra" ketone bodies are not produced by lactate, then one must infer that lactate yields ketone bodies at the expense of some other ketone body former. It is possible that this precursor may be pyruvate derived from the breakdown of tissue protein (see the accompanying scheme). Pyruvate may be considered as a major contributor to glucose formation in the phlorhizinized animal but *it also contributes to ketone body production.* If, as seems likely, it is decarboxylated to a 2-carbon intermediate, the resulting product can not only form intermediates of the tricarboxylic cycle but can theoretically be converted to fat, ketone bodies, and carbon dioxide. On this basis

*Proposed Metabolic Pathways of Lactate and Alanine*

administration of lactate will yield a large amount of "extra" glucose and simultaneously incorporate some of its isotope into ketone bodies.

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## SUMMARY

The metabolic fate of isotopic alanine and lactate in the phlorhizinized rat has been studied. Although 60 to 70 per cent of the administered alanine appears to be converted into "extra" urinary glucose, only 1 to 5 per cent of the administered isotope was recovered therein.

The administration of lactate labeled with  $\text{C}^{14}$  in the  $\alpha$  or  $\beta$  position results in the excretion of urinary glucose containing 28 per cent of the administered isotope. Approximately half of the radioactive carbon of the lactate was converted to fat and urinary ketone bodies, even though the anticipated amounts of "extra" urinary glucose were formed. All 4 carbons of the excreted acetoacetate and  $\beta$ -hydroxybutyrate were found to be radioactive. Evidence is presented that, to a significant degree, lactate may be directly converted by the phlorhizinized rat to ketone bodies without intermediate formation of fat.

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# THE INFLUENCE OF PYRIMIDINES UPON THE URINARY EXCRETION OF THIAMINE AND PYRAMIN\*.†

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The urinary excretion of thiamine and pyramin was studied in normal young men who were maintained on an essentially constant thiamine intake (4). It was noted that the small day to day variations in the urinary excretion of pyramin were not directly related to the slight daily changes in the dietary thiamine intake. That finding raised the question as to whether the pyrimidines related to thiamine present in the diets might be the cause of this variation. The food used in that study was composed primarily of preserved products such as dehydrated potatoes, canned meat, canned vegetables, and dried fruit. According to presently accepted theory, the treatment to which these foods were subjected results in the cleavage of the thiamine molecule with the liberation of the pyrimidine and thiazole groups. This could produce a variable pyrimidine content of the diet, depending upon the amount of thiamine that was destroyed during the processing and preparation of the food.

A second factor causing some concern in our pyramin studies was the occurrence of a so called "dietary blank." Urinary pyramin excretions for various levels of thiamine were secured after the subjects had become adjusted to the particular thiamine intake levels. Extrapolation of these values to a zero thiamine intake indicated an excretion of about 70  $\gamma$  per day (4). This "dietary blank" might conceivably be due to the presence of pyrimidines derived from the decomposition of thiamine in the diet.

The final factor which made these studies necessary was the finding that during an acute thiamine deficiency the urinary pyramin excretion after a few days of adjustment showed a progressive decrease to a level of 90  $\gamma$  per day. Stabilization of the pyramin excretion appeared to occur at that

\* Pyramin is the pyrimidine-like component of thiamine which is excreted in the urine.

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value even though some of the subjects showed clear signs of thiamine deficiency. The high pyrimin excretion might have been due to the diet. Only a few substances in that diet could be possible contributors of pyrimidine. Autoclaved yeast extract which was fed as a source of the B vitamins other than thiamine appeared to be the most likely source (1).

#### EXPERIMENTAL

Normal young men were the subjects in all of these experiments. They were maintained on different régimes of constant thiamine intake for at least 4 weeks before any urinary excretion studies were made. This was done in order to rule out any changes that might be ascribed to the period before stabilization occurred (4). In some of the experiments, the subjects received known amounts of 2-methyl-4-amino-5-ethoxymethylpyrimidine hydrochloride (hereinafter referred to as synthetic pyrimidine) in the form of pills and in others capsules containing dried yeast extract which had been treated in one of two ways.

24 hour urine samples were collected in bottles containing 5 ml. of toluene and 5 ml. of glacial acetic acid. In all cases the collection period was extended to include 2 or 3 days. Thiamine was determined in them by a modification of the thiochrome procedure (3) and pyrimin by a modification of the yeast fermentation method developed by Schultz, Atkin, and Frey (8) for the determination of thiamine. In this procedure, pyrimin is determined as the sulfite blank. Feces were collected for 4 day periods with carmine as a marker. These samples were analyzed for thiamine and pyrimin by the above methods.

Some of the diets used in the preceding experiments (4) were analyzed for the pyrimidine component of thiamine by means of the yeast fermentation method used in the determination of pyrimin (8).

In order to destroy the thiamine in the yeast extract it was autoclaved for 5 hours at 15 pounds pressure. As a check on the completeness of the thiamine destruction, the autoclaved yeast was analyzed for this vitamin by means of the thiochrome procedure. The pyrimidine component of thiamine in the autoclaved yeast was determined by means of the yeast fermentation method (8). The method of Obermeyer and Chen (5) was also used for the determination of this component of thiamine. In a separate experiment, the autoclaved yeast was hydrolyzed prior to analysis. This was done by refluxing it for 45 minutes with 0.2 N sulfuric acid. Both the autoclaved and the acid-hydrolyzed autoclaved yeast extracts were fed to the subjects in capsule form.

#### Results

Most of the diets that were analyzed contained from 80 to 100  $\gamma$  of the pyrimidine moiety expressed in terms of the synthetic pyrimidine.

When the synthetic pyrimidine was fed as pills at levels from 0.5 to 4.0 mg. per day, the urinary pyramin excretion increased only slightly over that of the preceding control period. In ten different experiments this increase averaged 2.5 per cent of the ingested pyrimidine dose in the four subjects (Tables I and II). The urinary recovery of the fed pyrimidine showed no

TABLE I

*Effect of Ingesting 2.5 Mg. of 2-Methyl-4-amino-5-ethoxymethylpyrimidine Hydrochloride Per Day upon Urinary Pyramin Excretion*

Each value is the mean of four to five 24 hour excretion values obtained after the subject had been maintained on constant thiamine intake for a month or more and is characterized by a variability of  $\sigma = 20.5 \gamma$  per day.

Subject	Experimental period		Difference	Per cent recovered
	Control	Pyrimidine		
Gl	147	216	69	2.8
No	142	193	51	2.0
Wa	175	228	53	2.1

TABLE II

*Effect of Ingesting Various Amounts of 2-Methyl-4-amino-5-ethoxymethylpyrimidine Hydrochloride upon Urinary Thiamine and Pyramin Excretion Levels*

All excretion values are the averages of approximately 1 week periods and are expressed as micrograms per day.

Subject		Synthetic pyrimidine intake, mg. per day				
		0	0.5	1.0	2.0	4.0
		Excretion				
Wa	Pyramin	141	168	154	181	250
	Thiamine	63	63		66	65
	$\Delta$ pyramin		27	13	40	109
	% recovery		5.4	1.3	2.0	2.7
No	Pyramin	159	175	183	222	
	Thiamine		198	186	200	
	$\Delta$ pyramin		16	24	63	
	% recovery		3.2	2.4	3.1	

change on a percentage basis when the pyrimidine intake varied from 0.5 to 4.0 mg. per day. The ratio between the molecular weight of the synthetic pyrimidine used in these studies and that for thiamine hydrochloride is 1:1.67. This means that the level of synthetic pyrimidine used as oral supplements in the above studies ranged from 0.8 to 6.7 mg. equivalents of thiamine.

The fecal excretion of pyramin-like substances decreased following the

feeding of 2.5 mg. of the synthetic pyrimidine per day. The decrease ranged from approximately 20 to almost 50 per cent of the original levels (Table III). The thiamine excretion in the feces under the same conditions also decreased by approximately 10 to 17 per cent below the original excretion level.

TABLE III

*Effect of Ingesting 2.5 Mg. of 2-Methyl-4-amino-5-ethoxymethylpyrimidine Hydrochloride upon Fecal Excretion of Thiamine and Its Pyrimidine Component Expressed in Terms of Synthetic Pyrimidine*

All values in micrograms per 24 hours. The excretion values are given for both the control period and that when the synthetic pyrimidine was added to the diet.

Subject		Control	Synthetic pyrimidine added	Difference
Gl	Pyrimidine component	47	28	-19
	Thiamine	1854	1540	-314
No	Pyrimidine component	40	21	-19
	Thiamine	2735	2265	-470
Wa	Pyrimidine component	47	38	-9
	Thiamine	2060	1842	-218

TABLE IV

*Effect of Ingesting 2.5 Mg. of 2-Methyl-4-amino-5-ethoxymethylpyrimidine Hydrochloride Per Day upon Urinary Excretion of Thiamine*

Each excretion value is the mean of two to three 24 hour values having a variation of  $\sigma = 30.3 \gamma$  per day. The changes are not significant.

Subject	Control	Synthetic pyrimidine added	Difference
Bi	225	262	+37
Pa	146	132	-14
Bo	315	290	-25
Re	205	186	-19
Pe	112	130	+18
Hu	136	116	-20

When the synthetic pyrimidine was fed to six young men at a daily level of 2.5 mg., there was no significant change in the urinary thiamine excretion (Table IV). The level of synthetic pyrimidine fed was equivalent to 4 mg. of thiamine. In spite of this large intake, the observed variations in urinary excretion were all within the range one would expect if these men had been maintained on a constant thiamine intake throughout the experiment (4).

Both the original yeast extract and the autoclaved sample were analyzed for thiamine by the thiochrome procedure and the autoclaved yeast extract

for the pyrimidine component of thiamine. The untreated yeast extract contained 0.455 mg. of thiamine per gm., whereas the autoclaved sample was free of this substance. If autoclaving had simply split the thiamine molecule into its pyrimidine and thiazole groups, then the autoclaved yeast should have contained 0.275 mg. of pyrimidine per gm. (expressed in terms of synthetic pyrimidine). Actual analysis for this compound by the yeast fermentation method showed the presence of 0.075 mg. of pyrimidine per gm. Only 27 per cent of the theoretical pyrimidine could be accounted for by this method. These values were secured when weighed amounts of the finely powdered autoclaved yeast extract were added directly to the reaction vessel in the fermentometer. When the autoclaved yeast extract

TABLE V

*Effect of Feeding Autoclaved Yeast Extract upon Urinary Excretion of Thiamine and Pyramin*

Each excretion value is the mean of three to six 24 hour urine samples which had variations of  $\sigma = 21.9 \gamma$  for pyramin and  $\sigma = 38.7 \gamma$  for thiamine. See the text for the explanation of "theory" and "analysis." The "yeast" period represents the excretion during the time that the autoclaved yeast extract was fed.

Subject		Urinary excretion			Pyrimidine fed		Recovery	
		Control	Yeast	Difference	Theory	Analysis	Theory	Analysis
		$\gamma$	$\gamma$	$\gamma$	$\gamma$	$\gamma$	per cent	per cent
Ge	Pyramin	295	361	+66	480	131	13.7	50.4
	Thiamine	334	579	+245				
No	Pyramin	166	180	+14	960	262	1.5	5.3
	Thiamine	114	112	-2				
Gl	Pyramin	156	211	+55	1200	327	4.6	16.8
	Thiamine	99	128	+29				
Ra	Pyramin	191	270	+79	1680	458	4.7	17.2
	Thiamine	175	240	+65				

was analyzed for total fermentation activity according to the original procedure (8), the apparent pyrimidine content was reduced to 0.055 mg. per gm. The latter procedure involved boiling for 20 minutes in a slightly acid solution and apparently destroyed about a fourth of the pyrimidine found in the autoclaved yeast extract by direct analysis. The method of Obermeyer and Chen (5) indicated the presence of 0.065 mg. of pyrimidine per gm.

When the autoclaved yeast extract was fed to normal young men, the urinary excretion of pyramin was quite unusual. In three of the four subjects, the increase in the pyramin excretion was approximately 3 per cent when calculated on the basis of the pyrimidine theoretically present in this supplement (Table V). On the basis of the pyrimidine found in the

autoclaved yeast extract by actual analysis, the urinary recovery was approximately four times higher than this. One of the subjects, Ge, showed results which were completely out of line with these. There is a possibility that this was an artifact. If subject Ge is omitted, then two of the remaining three subjects showed an increased thiamine excretion when the autoclaved yeast was fed, but these increases were all within the range of normal variability.

When the acid-hydrolyzed autoclaved yeast extract was fed to two young men, the urinary pyramin excretion increased to an extent which accounted for about 3 per cent of its theoretical pyrimidine content (Table VI).

TABLE VI

*Effect of Feeding Acid-Hydrolyzed Yeast Extract upon Urinary Excretion of Thiamine and Pyramin*

Each excretion value is the mean of two to five 24 hour urine samples which had variations of  $\sigma = 30.2$  for pyramin and  $\sigma = 69.9$  for thiamine. See the text for explanations of "theory" and "analysis." The "yeast" period represents the excretion during the time that the acid-hydrolyzed autoclaved yeast extract was fed.

Subject		Urinary excretion			Pyrimidine fed		Recovery	
		Control	Yeast	Difference	Theory	Analysis	Theory	Analysis
		$\gamma$	$\gamma$	$\gamma$	$\gamma$	$\gamma$	per cent	per cent
Ja	Pyramin	303	345	+42	960	70	4.4	58
	Thiamine	654	655	+1				
Wa	Pyramin	185	236	+51	1680	130	3.0	40
	Thiamine	95	118	+23				

On the basis of the pyrimidine found by actual analysis of the acid-hydrolyzed autoclaved yeast extract, the urinary recovery accounts for 50 per cent of that ingested. Again, the influence of the yeast on the urinary thiamine excretion was insignificant.

#### DISCUSSION

The urinary excretion of pyramin represents a variable fraction of the thiamine intake. When the thiamine intake is plotted against the pyramin excretion, the resulting curve shows an excretion of 70  $\gamma$  of pyramin at zero thiamine intake. The curve rises continuously to a thiamine intake of 2.5 mg. per day where the excretion levels off at a value of about 450  $\gamma$  of pyramin (4). At first the pyramin excretion at zero thiamine intake was considered as a "dietary blank," due to the pyrimidines contributed by the processed foods in the diets. This suggestion found confirmation in the 90  $\gamma$  of pyramin excretion actually observed when the subjects were maintained on a zero thiamine intake. This caused considerable concern since

the diets used in our studies (4) provided 80 to 100  $\gamma$  of pyrimidine per day. If this level of pyrimidine were responsible for the urinary "dietary blank," then any slight fluctuation in the amount present in the diet would constitute an important source of the variation observed in the daily pyrimin excretion. However, there was no consistent relationship between the pyrimidine content of the diet and the day to day variations in urinary pyrimin excretion.

Additional support for the hypothesis that the "dietary blank" was not primarily related to the diet was found in a consideration of the different diets used in our laboratory. In later experiments the diets contained mainly fresh food, while in the earlier experiments (4) they were made largely of processed foods. The amount of thiamine degradation products in these two cases was obviously very different; yet in spite of this, the pyrimin excretion data fitted the same intake-excretion curve. In order to account for a "dietary blank" of 70  $\gamma$  per day on the basis of the 3 per cent excretion observed in this work, it would be necessary to assume that the diets contained pyrimidine breakdown products derived from the cleavage of 4 mg. of thiamine. None of the diets contained that much thiamine originally.

A more plausible explanation for the pyrimin excretion at zero thiamine intake is that this represents the thiamine metabolism when no outside source of thiamine is available. It is impossible at present to indicate whether or not this should be considered a basic or special type of vitamin metabolism. This implies that another mechanism was also operative, according to which the urinary excretion of pyrimin is proportional within limits to the thiamine intake.

The present work indicates that, when a synthetic pyrimidine such as 2-methyl-4-amino-5-ethoxymethylpyrimidine is fed, only 3 per cent of the intake can be accounted for. Yet, in the normal metabolism of thiamine, a larger fraction of the intake must be excreted as the pyrimidine component in the form we have termed pyrimin. It is known that, besides the pyrimidine component of thiamine, fairly large amounts of the thiazole group are excreted in the urine.<sup>1</sup> This would make it plausible to assume that the metabolic breakdown of thiamine at some stage involves a split of the compound into the pyrimidine and thiazole groups. On this assumption, it might be expected that the excretion of the pyrimidine group would be similar when either thiamine or the synthetic pyrimidine is fed. Why there should be a marked difference in the recovery of the pyrimidine in these two cases is unknown.

At present it is impossible to explain why the increase in the pyrimin

<sup>1</sup> Johnson, B. C., personal communication.

excretion should represent about 3 per cent of the pyrimidine theoretically present in the autoclaved or acid-hydrolyzed autoclaved yeast extract. The relation of pyrimin excretion to pyrimidine fed is much more constant when calculated on the basis of the theoretical content than on the basis of that found by actual analysis of the treated yeast extract. The fate of most of the pyrimidine originally present in the yeast is unknown. Poor intestinal absorption, conversion of the pyrimidine to thiamine by the bacteria in the intestinal tract, and the loss of this thiamine either in the feces or in the urine have been eliminated as possible explanations for this loss.

The action of the synthetic pyrimidine in decreasing the excretion of both thiamine and pyrimin-like substances in the feces was unexpected. Originally, it was thought that some of the pyrimidine might have passed into the lower part of the gastrointestinal tract where it was converted by the bacteria into thiamine. This obviously does not happen; in fact, it appears as though the synthetic pyrimidine actually suppresses both the thiamine and the pyrimin-like compounds in the feces.

Although there is very little evidence on the ability of pyrimidines to replace thiamine, it is generally assumed that the pyrimidines are unable to act as substitutes for this vitamin in the metabolism of higher animals (9). Under the conditions of this experiment, it was impossible to secure a complete answer to this question; but the influence of one of these compounds on the urinary excretion of thiamine and pyrimin was studied. In the absence of any evidence to the contrary, it may be assumed that, if the pyrimidine were used by the body in place of dietary thiamine, then at least a fraction of the "spared" thiamine should appear in the urine. The ingestion of even large amounts of the synthetic pyrimidine had no essential influence on the urinary thiamine excretion, so that from this evidence alone it does not appear likely that this pyrimidine can replace thiamine as far as human beings are concerned.

There have been a number of suggestions that heat treatment of thiamine results in the breakdown of this compound into its pyrimidine and thiazole groups. This was based originally on studies made by Robbins and Bartley (6) who went no further than to get suggestive evidence for the presence of the pyrimidine group in autoclaved yeast. Schopfer and Müller (7) extended this type of work and claimed that thermal decomposition of thiamine resulted in the formation of both the pyrimidine and the thiazole moieties. More recently Obermeyer and Chen (5) have indicated that heat treatment of substances such as yeast, bread, flour, and crystalline thiamine produces a cleavage of the thiamine molecule. In these cases from 37 to 67 per cent of the destroyed thiamine could be accounted for as the pyrimidine and thiazole moieties. The method of Obermeyer and

Chen involves the incubation of the test sample with bakers' yeast and flour for 24 hours. The thiamine content of the above mixture was determined before and after incubation by means of the thiochrome technique. In order to determine the moiety which was present in limiting amount, other fermentation assays were made with added thiazole in one case and added synthetic pyrimidine in the other. Their data are difficult to interpret, but it appears that for the heat-treated substances the addition of either thiazole or pyrimidine produced an increase in thiamine above that secured in the absence of these compounds. According to their hypothesis, the incubation carried out without either one of the thiamine moieties should have been limited by only one of these compounds. Then when the sample was incubated with the thiamine moieties, only one of them should produce any increase in the thiamine content beyond that secured in the absence of these compounds. Obermeyer and Chen do not even mention this increased formation of thiamine by the addition of both thiamine moieties.

In only a few cases have the actual degradation products of thiamine been isolated. Williams and coworkers (10) isolated the sulfited pyrimidine and thiazole groups from a solution of the vitamin to which sodium bisulfite had been added. Krampitz and Woolley (2) isolated 4-methyl-5-hydroxyethylthiazole and 2-methyl-4-amino-5-hydroxymethylpyrimidine as products resulting from the action of thiaminase on crystalline thiamine. Until chemical isolation studies similar to these are applied to the degradation products of thiamine resulting from heat treatment, it is very difficult to get any true indication of the actual reactions. The use of biological methods in answering the question of the products formed is of dubious value, since the specificity of most of the methods has not been adequately established.

#### SUMMARY

1. When 2-methyl-4-amino-5-ethoxymethylpyrimidine was given to normal young men at levels up to 4 mg. per day, only 3 per cent of the intake could be recovered in the urine as pyramin during the following 24 hours. The fraction recovered was constant over an 8-fold range of intake levels. The pyrimidine has no influence on the urinary excretion of thiamine. There was a considerable decrease in the fecal excretion of both thiamine and pyramin following the feeding of the pyrimidine. On this basis, there is no evidence that this pyrimidine can substitute for thiamine in man.

2. When autoclaved yeast extract was fed, the urinary recovery represented 3 per cent of the pyrimidine theoretically resulting from the destruction of the thiamine originally present in the extract. A maximum of 27



per cent of this pyrimidine could be accounted for by direct analysis of the autoclaved yeast extract. Boiling the autoclaved yeast extract in dilute acid apparently destroyed about a fourth of that detected by direct analysis. This would indicate the need for caution in the preparation of samples for thiamine analysis by the yeast fermentation method.

3. When acid-hydrolyzed autoclaved yeast extract was fed, 3 per cent of the theoretical pyrimidine was again recovered as pyramin in the urine.

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## PEPTIDE BOND SYNTHESIS

### III. ON THE MECHANISM OF *p*-AMINOHIPPURIC ACID SYNTHESIS\*

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By the study of *p*-aminohippuric acid (PAH) synthesis in rat liver homogenates (1), it was established that the reaction was in some manner related to the transfer of high energy phosphate. However, even with the dilution of endogenous metabolites by the homogenization technique, the high rate of synthesis obtained in the absence of any substrate other than *p*-aminobenzoic acid (PAB) and glycine prevented precise evaluation of the mechanism by which energy is transferred from the oxidative metabolism to the synthetic system. This was particularly true of the rôle of the adenylic phosphates.

We have now found that the synthetic activity is associated with the insoluble residues of the rat liver cell, which has enabled us to suppress almost completely the endogenous metabolism by differential centrifugation and washing. In unpublished work with entire homogenates, we had found adenylic acid to be as active as adenosine triphosphate (ATP) in stimulating the formation of PAH, which had led us to conjecture that adenylic acid might be the active agent, functioning in a hitherto unknown manner. We now know that this is not true, that ATP is the actual energy-donating compound, and that adenylic acid is effective only under conditions by which it may be phosphorylated to form ATP.

This paper reports the experiments establishing the above facts, together with data on some properties of the enzyme system.

#### EXPERIMENTAL

*Tissues*—Isotonic KCl homogenates were made as before (1). A portion of the homogenate was centrifuged at 2000*g* for 10 minutes in the cold. The sediment obtained by this treatment should contain all of the cell nuclei together with a part of the mitochondrial fraction (2) and is designated in this paper as the *residue*. For washing, the residue was resuspended in the original volume of fresh KCl, and centrifuged as above. Residue washed twice in this manner is designated *washed residue*. Other treatments are described in the individual experimental details.

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*Substrates*—Adenylic acid was prepared from ATP by the method of Kerr (3). N-Phosphoglycine was prepared by the method of Winnick and Scott (4). Other substrates were obtained as previously reported (1). The incubation media were prepared by mixing isotonic solutions of the substrates at pH 7.55, and usually contained 0.0026 M magnesium sulfate, 0.04 M potassium chloride, 0.001 M PAB, and 0.015 M glycine and were made isotonic with potassium phosphate at pH 7.55 after addition of any further substrates, so that the final phosphate ion concentration varied from 0.04 to 0.06 M.

*Procedure*—The incubation and analysis were carried out as before (1). The results are expressed as micromoles of PAH formed per mg. of tissue nitrogen.

### Results

*Differential Centrifugation*—In these initial experiments, adenylic acid rather than ATP was added to the media. Since the residue contains all of the nuclei and has 80 per cent of the activity (Table I), while the supernatant has less than 4 per cent, and addition of the boiled supernatant to the residue results in almost complete restoration of the original activity, it is apparent that the activity is associated with the large insoluble components of the cell. In other experiments, in which the supernatant was added to the washed residue, 84 per cent of the activity of the entire homogenate was attained, showing that, at most, 16 per cent was lost in the washing procedure. These observations are complicated by an initial rise in the activity of the homogenate upon storage in the cold. At the end of 4 hours, the activity is 6 to 15 per cent greater, although at the end of 24 hours storage it has dropped to 37 to 45 per cent of the initial value. Since the residue activity is compared to the activity of homogenate which has been stored during the separation and washing procedures, the results are affected by changes within the entire homogenate which may not be occurring in the separated residue.

*Cometabolites*—The effect of removing the endogenous metabolites was immediately apparent in the experiments of Table II. If either, or both, fumarate or adenylic acid was removed from the media, the activity was 8 to 12 per cent that of the complete system. Removing cytochrome *c* reduced the activity to 23 per cent. None of these substances showed as clear an action in the entire homogenate (1).

Magnesium ion was also clearly required (Fig. 1), although it stimulated only a few per cent in the entire homogenate. Instead of the inhibiting action formerly experienced at higher concentrations, a second plateau was now reached.

*Adenosine Phosphates*—It was now possible critically to examine the

rôle of the adenylic system. ATP was markedly more effective than adenylic acid at all concentrations (Fig. 2). In experiments extending

TABLE I  
*Differential Centrifugation of Rat Liver Homogenate*

15 per cent rat liver homogenate was centrifuged at 2000*g* for 10 minutes. Both the supernatant and residue were made up to the original volume with isotonic KCl. Boiled supernatant was prepared by heating the supernatant 5 minutes at 100°, centrifuging off the precipitated proteins, and using the clear yellow solution. Tissue nitrogen per flask, entire homogenate 3.48 mg., residue 1.13 mg., supernatant 2.27 mg. Final concentrations, 0.001 *M* *p*-aminobenzoic acid, 0.015 *M* glycine, 0.0004 *M* magnesium sulfate, 0.0025 *M* fumarate, 0.0005 *M* adenylic acid, 0.000012 *M* cytochrome *c*, 0.04 *M* potassium chloride. Total volume of 4.0 ml. made isotonic with potassium phosphate at pH 7.55, and incubated 40 minutes at 38° with air as the gas phase.

Tissue	PAH formed	
	<i>micromoles</i>	<i>micromoles per mg. N</i>
Entire homogenate	1.98	0.57
Residue	1.59	1.41
“ + supernatant	1.98	0.57
Supernatant	0.07	0.03
Residue + boiled supernatant	1.96	1.73

TABLE II  
*Effect of Fumarate, Adenylic Acid, and Cytochrome c*

1.0 ml., containing 1.28 mg. of nitrogen, of washed residue suspension added per flask. Final concentrations, 0.001 *M* *p*-aminobenzoic acid, 0.015 *M* glycine, 0.0004 *M* magnesium sulfate, 0.04 *M* potassium chloride. Final volume of 4.0 ml. made isotonic with potassium phosphate at pH 7.55, and incubated 40 minutes at 38° with air as the gas phase.

Substrates			PAH formed <i>micromoles per mg. N</i>
Fumarate, 0.0025 <i>M</i>	Cytochrome <i>c</i> , 0.000012 <i>M</i>	Adenylic acid, 0.0005 <i>M</i>	
+	—	—	0.10
—	+	—	0.09
—	—	+	0.12
+	+	—	0.10
+	—	+	0.27
—	+	+	0.13
+	+	+	1.13

the concentration range, it was found that adenylic acid began to inhibit beyond 0.0005 *M*, the value at 0.0015 *M* being 78 per cent that of the peak value, while the ATP curve was essentially flat over the same range.

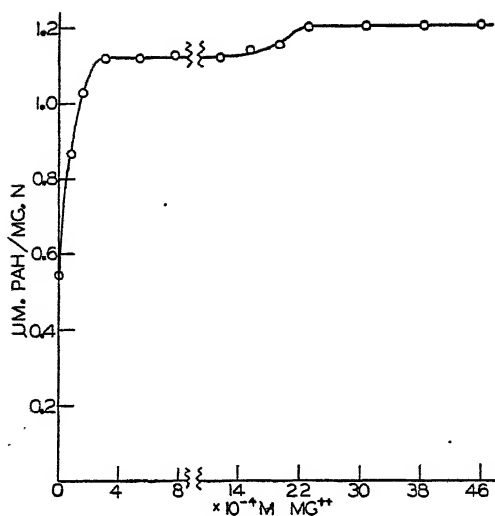


FIG. 1. The effect of magnesium ion. 1.0 ml., containing 1.04 mg. of nitrogen, of washed residue suspension added per flask. Final concentrations, 0.001 M *p*-amino-benzoic acid, 0.015 M glycine, 0.0005 M adenylic acid, 0.0025 M fumarate, 0.000012 M cytochrome c, 0.04 M potassium chloride; the total volume of 4.0 ml. made isotonic with potassium phosphate at pH 7.55; incubated 40 minutes at 33° with air as the gas phase.

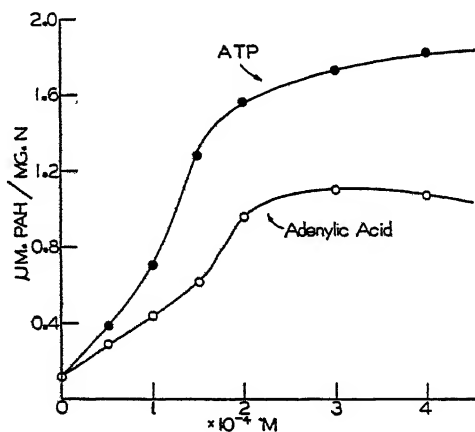


FIG. 2. Comparison of adenylic acid and adenosine triphosphate (ATP). 1.0 ml., containing 0.82 mg. of nitrogen, of washed residue suspension added per flask. Final concentrations, 0.001 M *p*-aminobenzoic acid, 0.015 M glycine, 0.0025 M fumarate, 0.000012 M cytochrome c, 0.0026 M magnesium sulfate, 0.04 M potassium chloride; the total volume of 4.0 ml. made isotonic with potassium phosphate at pH 7.55; incubated 40 minutes at 33° with air as the gas phase.

In the absence of any added oxidative metabolite, ATP could support the reaction anaerobically (Fig. 3), in contrast to adenylic acid (Table II). The endogenous metabolism is not completely suppressed in the washed residue, as is shown by the difference between the aerobic and anaerobic curves at higher ATP concentrations. It may be noted in comparing Figs. 2 and 3 that the addition of the oxidative system, fumarate plus cytochrome *c*, to resynthesize ATP hydrolyzed by adenosinetriphosphatase (ATPase) is desirable to avoid the use of high ATP concentrations. The shape of Fig. 2 is complex, due to the balance between synthesis and

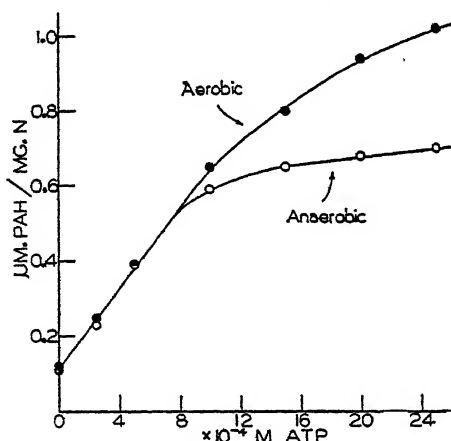


FIG. 3. Comparison of aerobic and anaerobic conditions on adenosine triphosphate (ATP) activity. 1.0 ml., containing 1.03 mg. of nitrogen, of washed residue suspension added per flask. Final concentrations, 0.001 M *p*-aminobenzoic acid, 0.015 M glycine, 0.0026 M magnesium sulfate, 0.04 M potassium chloride; the total volume of 4.0 ml. made isotonic with potassium phosphate at pH 7.55; incubated 40 minutes at 38°; aerobic flasks incubated with air, anaerobic with nitrogen, as the gas phase.

hydrolysis of ATP, while that of Fig. 3, where little synthesis occurred, is relatively simple.

The experiment of Table III demonstrated two interesting points. Storing the entire homogenate in the cold for the length of time necessary to complete six washings of the residue (90 minutes) resulted in almost complete loss of activity in the absence of added ATP, whereas in our previous study (1) we had been able to demonstrate only a slight effect, employing fresh homogenates. Secondly, the loss of activity upon repeated washings appears to be due largely to loss of ability to resynthesize ATP, since the activity with the latter compound alone is relatively constant, although the amount of synthesis attained with the complete system drops 44 per cent during six washings. The activity per mg. of

tissue nitrogen reaches its peak upon four washings. It is to be remembered in this connection that the efficacy of the separation depends upon the insoluble material being intact or in large fragments. The continued slow drop in the nitrogen value of the residue upon redispersion attests the aggravation of the original damage done by homogenization. After four washings the loss of synthetic activity exceeds the loss of competing systems.

TABLE III

*Effect of Washing on Residue Activity*

Residue washed two, four, and six times with isotonic KCl, and made up to original homogenate volume with isotonic KCl. Tissue nitrogen per flask, entire homogenate 3.18 mg., twice washed residue 0.97 mg., residue washed four times 0.85 mg., residue washed six times 0.76 mg. Final concentrations, 0.001 M *p*-aminobenzoic acid, 0.015 M glycine, 0.04 M potassium chloride, 0.0026 M magnesium sulfate. Total volume of 4.0 ml. made isotonic with potassium phosphate at pH 7.55, and incubated 40 minutes at 38° with air as the gas phase.

Tissue	Substrates		PAH formed	
	0.0025 M fumarate + 0.000012 M cytochrome <i>c</i>	0.001 M ATP	micromoles	micromoles per mg. N
Entire homogenate	—	+	0.62	0.19
	+	—	0.09	0.03
	+	+	2.32	0.73
Twice washed residue	—	+	0.67	0.69
	+	—	0.12	0.12
	+	+	1.59	1.64
Residue washed 4 times	—	+	0.65	0.76
	+	+	1.58	1.86
Residue washed 6 times	—	+	0.61	0.81
	+	+	1.31	1.73

To test for possible phosphorylated intermediates, the experiment of Table IV was devised. N-Phosphoglycine is evidently not an intermediate. Since the rate of the reaction is less if glycine is not present during the first 20 minutes of incubation, there must not be any accumulation of an intermediate *p*-aminobenzoyl phosphate. Although this does not exclude the transient formation of an intermediate, it is probable that none has an independent existence. The close agreement of the sum of the PAH formed in the first and second halves of the incubation period with that formed during the entire period precludes significant loss in activity due to the absence of one substrate during the first half of the incubation.

*Coenzymes*—The pyridine nucleotides, thiamine pyrophosphate, or pyridoxal phosphate did not have any marked effect on the rate of synthesis

TABLE IV  
*Test for Intermediates*

1.0 ml., containing 0.84 mg. of nitrogen, of washed residue suspension added per flask. Final concentrations, 0.001 M *p*-aminobenzoic acid, 0.0075 M magnesium sulfate, 0.04 M potassium chloride, 0.0025 M fumarate, 0.000012 M cytochrome *c*. Total volume of 4.0 ml. made isotonic with potassium phosphate at pH 7.55; N-phosphoglycine added as magnesium salt; substrates not present during the first half of incubation added from the side arm at 20 minutes, and incubated 40 minutes at 38° with air as the gas phase.

Substrate	Amount of PAH formed with substrate present		
	0 to 40 min.	0 to 20 min.	20 to 40 min.
	micromoles per mg. N	micromoles per mg. N	micromoles per mg. N
0.005 M glycine + 0.0005 M ATP	0.67	0.38	0.31
0.005 " N-phosphoglycine + 0.0005 M ATP	0.26	0.20	0.10
0.005 " "	0.05		

TABLE V  
*Coenzymes and Oxygen Consumption*

1.0 ml., containing 1.60 mg. of nitrogen, of washed residue suspension added per flask. Final concentrations, 0.04 M potassium chloride, 0.0026 M magnesium sulfate in all flasks; 0.001 M *p*-aminobenzoic acid (PAB), 0.0005 M adenosine triphosphate (ATP), 0.015 M glycine in all except endogenous metabolism flasks; 0.0025 M fumarate, 0.000012 M cytochrome *c* in all except substrate metabolism flasks. Total volume of 4.0 ml. made isotonic with potassium phosphate at pH 7.55 and incubated 40 minutes at 38° with air as the gas phase.

Substrates	PAH formed	Oxygen uptake
	micromoles per mg. N	microliters per mg. N
(a) None		6.5
(b) PAB, glycine, ATP	0.21	8.0
(c) (b) + fumarate, cytochrome <i>c</i>	1.20	74.0
(d) (c) + 0.0005 M diphosphopyridine nucleotide	1.15	87.1
(e) (c) + 0.00005 M triphosphopyridine nucleotide	1.22	82.7
(f) (c) + 0.0005 M thiamine pyrophosphate	1.25	78.1
(g) (c) + 0.00005 M pyridoxal phosphate	1.19	81.2

(Table V), but they did affect the oxygen consumption. No correlation of PAH synthesis with the rate of oxygen consumption can be noted.

The dilution curve with washed residue (Fig. 4) has the same shape as that found with whole homogenates (1) in the absence of ATP, except that



the slight dilution effect has disappeared, a linear extrapolation intersecting the axis at less than 0.03 mg. of residue nitrogen. This indicates that the system is complete, and if any cofactor is involved, it must be so strongly bound to the enzyme that the washing procedure does not remove it.

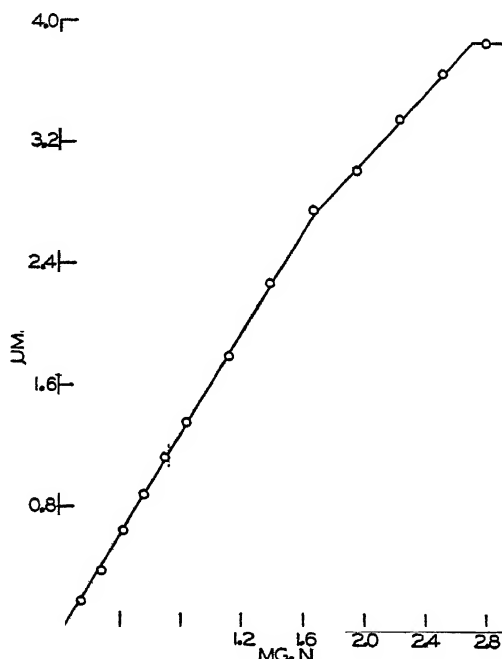


FIG. 4. Effect of washed residue concentration. Final concentrations, 0.001 M *p*-aminobenzoic acid, 0.015 M glycine, 0.0025 M fumarate, 0.000012 M cytochrome *c*, 0.0005 M adenosine triphosphate, 0.0026 M magnesium sulfate, 0.04 M potassium chloride; the total volume of 4.0 ml. made isotonic with potassium phosphate at pH 7.55; incubated 40 minutes at 38° with air as the gas phase.

*Stability of Enzyme*—We had early noted that freezing of the entire homogenate destroyed 85 per cent of the activity.

Fig. 5 illustrates the loss of activity upon making the incubation medium either hypo- or hypertonic by removal or addition of KCl. At 3.9 times the isotonic concentration all of the nucleoprotein, which usually forms fibrous masses during the incubation, had been converted to a viscous mass characteristic of desoxyribonucleoprotein solution in high salt concentrations (5). Changes in the state of aggregation of the nucleoprotein due to changes in tonicity caused irreversible inactivation of the system (Table VI) in which the washed residue was treated for 10 minutes with water or

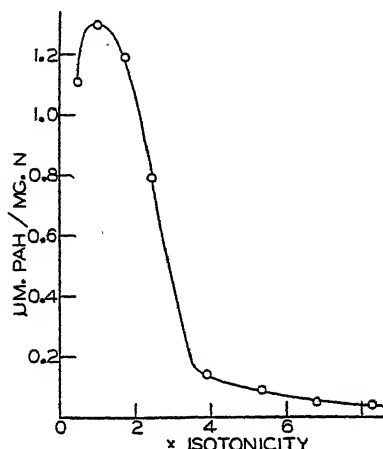


FIG. 5. Effect of osmotic pressure. 1.0 ml., containing 1.30 mg. of nitrogen, of washed residue suspension added per flask. Final concentrations, 0.001 M *p*-aminobenzoic acid, 0.015 M glycine, 0.0005 M adenosine triphosphate, 0.0025 M fumarate, 0.000012 M cytochrome *c*, 0.0026 M magnesium sulfate, 0.054 M potassium phosphate at pH 7.55; osmotic pressure expressed in fraction of isotonic concentrations and varied by removal or addition of KCl; incubated 40 minutes at 38° with air as the gas phase.

TABLE VI  
*Stability of Enzyme*

Washed residue aliquots treated with cold acetone and dried *in vacuo*, triturated 10 minutes with water, or triturated 10 minutes with 0.616 M KCl, and added as suspensions in KCl. Tissue nitrogen per flask, untreated washed residue 0.66 mg., acetone-dried residue 1.06 mg., water-treated residue 0.82 mg., KCl-treated residue 0.78 mg. Final concentrations, 0.001 M *p*-aminobenzoic acid, 0.015 M glycine, 0.0025 M fumarate, 0.0005 M adenosine triphosphate, 0.0026 M magnesium sulfate, 0.000012 M cytochrome *c*. Total volume of 4.0 ml. made isotonic with potassium phosphate at pH 7.55 before the addition of tissue (see the text), and incubated 40 minutes at 38° with air as the gas phase.

Treatment of residue	PAH formed
	<i>micromoles per mg. N</i>
None .....	0.97
Acetone-dried .....	0.04
Water trituration .....	0.28
0.616 M KCl trituration .....	0.67

with 0.616 M KCl and then added to the isotonic medium. The water solution was restored to isotonicity before addition to the medium, but the hypertonic suspension could not be diluted to isotonicity and still main-

tain the volume relationships in the final mixture; so it was incubated in medium which was 1.5 times isotonicity. While the short exposure to hypertonic KCl at 0° was inadequate to produce any visible solution of nucleoprotein, the amount of synthesis was 69 per cent of the control as compared to the 91 per cent achieved on incubation with 1.75 times isotonic solutions (Fig. 5), showing that the loss is not due to the exposure to a hypertonic concentration during incubation.

Acetone drying of the residue resulted in 96 per cent loss of activity. Apparently, any treatment affecting the association of the nucleoproteins destroys the enzyme system.

TABLE VII  
*Activity of Various Rat Tissues*

1.0 ml. of washed residue added per flask. Tissue nitrogen per flask, kidney 0.86 mg., brain 0.86 mg., testis 0.69 mg., spleen 0.63 mg., heart 0.51 mg., liver 1.63 mg. Final concentrations, 0.001 M *p*-aminobenzoic acid, 0.015 M glycine, 0.0025 M fumarate, 0.00012 M cytochrome *c*, 0.0005 M adenosine triphosphate, 0.0026 M magnesium sulfate, 0.04 M potassium chloride. Total volume of 4.0 ml. made isotonic with potassium phosphate at pH 7.55, and incubated 40 minutes at 38° with air as the gas phase.

Tissue	PAH formed
	<i>micromoles per mg. N</i>
Kidney.....	1.21
Brain.....	0.01
Testis.....	0.00
Spleen.....	0.00
Heart.....	0.03
Liver.....	1.13

*Occurrence of Enzyme*—As was found in tissue slices (6), only the rat liver and kidney possess activity (Table VII), and in the homogenate-washed residue, where diffusion is not a consideration as it is in slices, both tissues were approximately equally active. In interpreting these data from a quantitative standpoint, one should be cautioned that results with washed residue do not show the constancy from preparation to preparation experienced with entire homogenates. The variability may arise from slight differences in the condition of the animal and in the homogenization technique which may affect the distribution of the intracellular components upon centrifugation, as well as from uncontrolled differences in the washing technique.

#### DISCUSSION

ATP has been shown by Lipmann to be necessary for the formation of acetylated aromatic amines (7) and by Speck for the formation of glutamine

(8). These syntheses are closely related in that the enzymes involved may be extracted from an acetone powder of the soluble cellular material, in the requirements for magnesium ion, and in the formation of a hydroxamic acid in the presence of hydroxylamine. (Indeed, the latter observation would suggest that the formation of glutamine and the hydroxamic analogue may be a non-enzymatic reaction resulting from the phosphorylation of the "fatty acid" carboxyl group of glutamic acid, in the same manner that Lipmann has shown that the hydroxamic acids may be formed from the fatty acids. However, Lipmann established the fact that the acetylation of the aromatic amines is itself an enzymatic reaction which does not proceed through the acyl phosphate.)

The formation of PAH differs from the above reactions in that it is catalyzed by a system present in the insoluble residue of the cell which is sensitive to deviations in salt concentration and which requires potassium ion. It is remarkable, therefore, that it also proceeds by the transfer of high energy phosphate from ATP in the presence of magnesium ion.

The many mechanisms proposed for peptide synthesis include the reversal of proteolysis (9), transacylation of acetylated amino acids (10), the formation of keto peptides (11), the formation of dehydropeptides (12), and transphosphorylation (13). It is now recognized by most workers that the experimental coupling of protein synthesis to the oxidative metabolism excludes reversal of proteolysis as being the dominant mechanism for peptide bond formation. Further evidence excluding any mechanism involving the formation of acetylated amino acids, dehydropeptides, or keto peptides is provided through the work of Simmonds, Tatum, and Fruton (14) on mutant strains of *Escherichia coli* which required phenylalanine or tyrosine for growth. None of the derivatives listed above of the required amino acid was able to maintain growth, although the true peptides, as well as the free keto acids, could do so. In the absence of any more likely suggestion, we believe that future efforts should be directed along the lines of transphosphorylation, with ATP as the phosphate donor. Spiegelman and Kamen (15) have suggested nucleic acids as phosphate donors in protein synthesis.

A comparison of the data of this paper with that obtained with whole homogenates (1) emphasizes certain points of the homogenate technique. Since this system requires ATP, and the entire homogenate is able to carry on a fair degree of synthesis in the absence of any other substrates than PAB and glycine, it is obvious that the ATPase activity of homogenates is not able to keep the ATP concentration at ineffective levels. Admittedly, the concentration present at any instant is small and may appear in high energy phosphate determinations to be negligible, but a consideration of the maximum ATP concentration necessary when resynthesis is possible

(Fig. 2) will show that the effective level is small. Further, the concentrations of oxidizable metabolites and cytochrome *c* are anything but negligible. Even after washing the residue twice, enough residual metabolites are present to show a difference between the aerobic and anaerobic values at high ATP concentrations (Fig. 3). The maximum stimulation by cytochrome *c* obtained in the entire homogenate was 40 per cent. In the washed residue, the stimulation was 430 per cent. It is patent that the entire homogenate contains an appreciable cytochrome *c* concentration. The same is true of magnesium ion, whose slight variable effect in the previous study had led us to conclude that it was not necessary for the synthetic reaction.

We have avoided attaching a name to our enzyme system, believing it unwise to apply a name when the specificity and simplicity of the system are unknown. However, since the field of synthetic enzymes is beginning to be explored, we wish to suggest the term "synthetase" for those enzymes creating a new molecule by the elimination of water between two substrate molecules, excluding the formation of phosphate esters and anhydrides, and to precede the term with the name of the compound or class of compounds formed. Thus, our enzyme, if specific for PAH formation alone, would be "*p*-aminohippuric acid synthetase;" if specific for all hippuric acid syntheses, it would be "hippuric acid synthetase" and if general for all peptides, "peptide synthetase." The generic term, synthetase, would apply to all enzymes forming amides or esters. By this method, choline acetylase (16) would be given a name, depending upon the specificity of the acetylation, similar to "acetylcholine synthetase" or "acetate ester synthetase."

#### SUMMARY

1. The enzyme system forming *p*-aminohippuric acid (PAH) from *p*-aminobenzoic acid (PAB) and glycine has been found to be associated with the insoluble large particles of the rat liver cell.
2. The reaction requires magnesium ion and adenosine triphosphate (ATP).
3. Adenylic acid cannot replace ATP in the absence of an oxidizable substrate and is less effective than ATP under equivalent conditions.
4. ATP is more effective when fumarate and cytochrome *c* are added to regenerate the hydrolyzed ATP.
5. Diphosphopyridine nucleotide, triphosphopyridine nucleotide, thiamine pyrophosphate, or pyridoxal phosphate has only slight effect on the rate of synthesis.
6. The enzyme system is unstable to freezing, acetone drying, and hypo- or hypertonic salt concentrations.
7. The enzyme system occurs only in the liver and kidney of the rat.

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# THE SPECIFICITY OF ADENOSINE DEAMINASE AND PURINE NUCLEOSIDASE

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In the course of studies on the metabolism of purine nucleosides (1), we had the opportunity to examine two rare compounds of this class, adenine thiomethylpentoside and isoguanosine (crotonoside, 2-hydroxy-6-aminopurine-D-riboside). These substances afforded a chance to gain more insight into the rôle which substituents of the purine nucleus and of the carbohydrate part play in determining the substrate specificity. The following is a report of our findings with adenosine deaminase and purine nucleosidase.

## EXPERIMENTAL

*Substrates*—Adenine thiomethylpentoside (formula IV) was obtained from yeast<sup>1</sup> by a modification<sup>2</sup> of the method described by Levene (2). The well crystallized product (m.p. 211°) had the following elementary composition.

$C_{11}H_{16}O_5N_5S$	Calculated.	C 44.44, H 5.08, N 23.55, S 10.78
297.1	Found. <sup>3</sup>	" 44.22, " 5.12, " 23.46, " 10.98

The structure proposed by Wendt (3) agrees with that suggested by Suzuki, Otake, and Mori (4). Falconer and Gulland have found that the sugar is attached at position 9 of the adenine molecule (5). Hypoxanthine thiomethylpentoside was prepared from adenine thiomethylpentoside by nitrous acid treatment according to the directions of Kuhn and Henkel (6).

Isoguanosine (crotonoside) was discovered by Cherbuliez and Bernhard (7) in croton beans (*Croton tiglium* L.) and the structure as suggested by these authors was established beyond doubt by Spies and Drake (8). The attachment of the carbohydrate group in position 9 of the purine nucleus, as given in formula III, has been suggested by Falconer, Gulland, and Story (9). The source material for the preparation of crotonoside is almost unobtainable at present. The examination of this compound was

<sup>1</sup> Generously supplied by Anheuser-Busch, Inc., St. Louis.

<sup>2</sup> Schlenk, F., to be published.

<sup>3</sup> Dr. H. R. Morris, Galveston.



made possible by Dr. J. R. Spies who provided us with a generous sample of it.

Adenosine, xanthosine, and guanosine were prepared by the usual procedures (10).

*Enzymes*—Adenosine deaminase was prepared from small intestine by the directions of Brady (11) with additional purification steps. While this work was in progress, a new procedure was published by Kalckar (12) which is based on Schmidt and Thannhauser's earlier outline (13). Both methods yield preparations which, under suitable conditions, deaminate several times their own weight of adenosine per minute. A detailed account of our purification experiments will appear later. Nucleosidase was prepared from acetone-dehydrated rat or rabbit liver. The extract of this was purified according to Klein (14) and to Kalckar (12).

TABLE I  
*Action of Adenosine Deaminase on Various Substrates*

Experimental conditions as given in the text. Concentration of deaminase, 35.4  $\gamma$  per sample in Experiment A, 3540  $\gamma$  per sample in Experiment B.

Substrate	NH <sub>3</sub> liberated	
	Experiment A	Experiment B
	micromoles	micromoles
No substrate.....	<0.05	<0.05
Adenosine.....	37.05	35.90
Guanosine.....	<0.05	0.23
Isoguanosine.....	<0.05	<0.05
Adenine thiomethylpentoside.....	<0.05	<0.05

*Experiments with Adenosine Deaminase*—To study the rate of deamination both the conventional ammonia determination and the spectrophotometric procedures of Kalckar (12) were used.

In the former experiments the conditions were as follows: To 10 mg. of nucleoside in 1.0 ml. of 0.06 M phosphate buffer, pH 6.5, 1.0 ml. of enzyme solution was added and the mixture incubated at 37° for 2 hours. The reaction was stopped with 1.0 ml. of 1 N hydrochloric acid and the ammonia liberated was determined by distillation with an excess of alkali (15) and collection in a measured amount of 0.01 N hydrochloric acid. Control experiments showed that no significant amount of ammonia was liberated from the substrate or enzyme alone during incubation and distillation. In Table I the results of deamination experiments are shown. As can be seen, adenosine deaminase is strictly specific toward adenosine. This has been confirmed by spectrophotometric measurements. The use of spectrophotometry to follow enzymatic changes of purine compounds

was first described by Kalckar (12). We have applied his technique to our studies. The changes which would result from deamination of isoguanosine to xanthosine and of adenine thiomethylpentoside to hypoxanthine thiomethylpentoside are illustrated in Figs. 1 and 2. We found the absorption spectrum of isoguanosine rather different from that reported for this compound by Falconer, Gulland, and Story (9). There is close agreement between our data and those of these English authors with respect to adenine thiomethylpentose (5) and xanthosine (9). No data have been reported

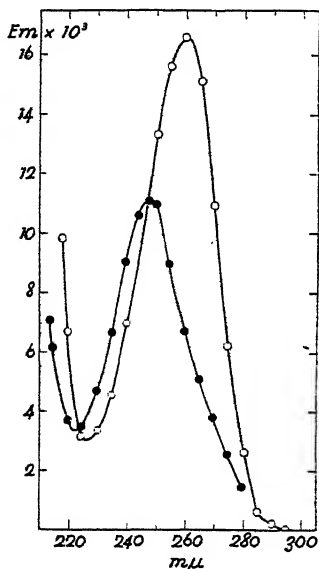


FIG. 1

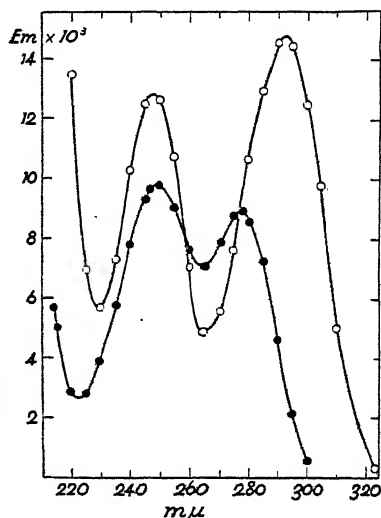


FIG. 2

FIG. 1. Absorption spectrum of adenine thiomethylpentoside (O) and hypoxanthine thiomethylpentoside (●), in 0.02 M phosphate buffer, pH 7.5.

FIG. 2. Absorption spectrum of isoguanosine (O) and xanthosine (●) in 0.02 M phosphate buffer, pH 7.5.

earlier for hypoxanthine thiomethylpentoside. Its absorption spectrum resembles that of inosine (16). A Beckman spectrophotometer, model DU, was used for the measurements which are given in Table II. Deamination of adenosine is indicated by the decrease in the absorption intensity at 260 mμ. Isoguanosine and adenine thiomethylpentoside are not attacked. Their absorption spectra remain unchanged.

*Experiments with Nucleosidase*—For determination of nucleosidase activity the iodometric procedures of Grynberg (17) and Dmochowski (18) as adapted by Klein (14) were used. Nucleosides do not react with hypoiodite, but, after splitting them into their components, the aldehyde

group and some of the purine bases react with iodine. Guanine reacts with 4 equivalents of iodine, while hypoxanthine reacts with about 1 equivalent. Adenine does not react under the conditions of the Willstätter-Schudel titration (19). Data for isoguanosine and isoguanine have not yet been listed in the literature. We found that isoguanosine does not reduce hypiodite, while isoguanine, like guanine, reacts with 4

TABLE II

*Action of Deaminase on Adenosine and Related Purine Nucleosides.  
Spectrophotometric Technique*

Concentration of substrates, 16.6  $\gamma$  per ml., deaminase 2.0  $\gamma$  per ml. of 0.066 M phosphate buffer, pH 7.5.

Substrate	Wave-length  <i>m<math>\mu</math></i>	Density readings after incubation for			
		0 min.	3 min.	12 min.	22 min.
Adenosine	260	0.729	0.600	0.515	0.485
Isoguanosine	290	0.780	0.777	0.772	0.770
Adenine thiomethylpentoside	260	0.722	0.722	0.722	0.722

TABLE III

*Action of Nucleosidase on Purine Nucleosides*

Each sample contained 17 micromoles of substrate and 8.7 mg. of nucleosidase in 3.5 ml. of 0.02 M phosphate buffer, pH 7.5. Incubation at 37°. Perchloric acid was used for deproteinizing (20).

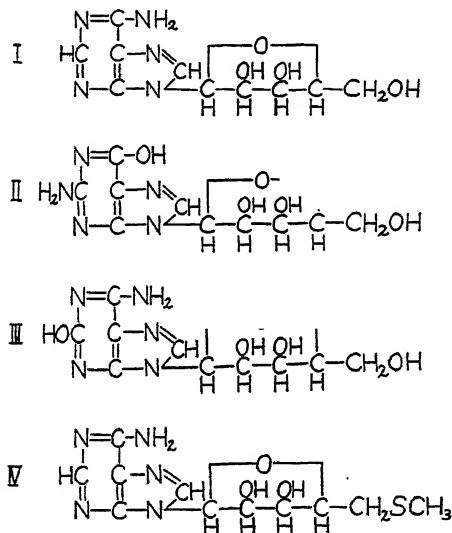
Substrate	Increase in microequivalents of iodine reduced after	
	1 hr.	3 hrs.
No substrate.....	0	0
Guanosine.....	10.2	29.1
Isoguanosine.....	1.3	2.1
Adenine thiomethylpentoside.....	0	0
Hypoxanthine thiomethylpentoside.....	0	2.5

equivalents of iodine. Therefore, the splitting of isoguanosine may be followed by the iodometric technique as in the case of guanosine.

The conditions are more complicated with adenine thiomethylpentoside. Wendt (3) has found that the thiomethyl group reduces 2 equivalents of iodine and yields the corresponding sulfoxide. He used 0.1 N iodine and alkali solution. Using more dilute solutions (0.01 N iodine and 0.02 N alkali), we found that less than 2 equivalents of iodine are bound by adenine and hypoxanthine thiomethylpentoside. Regardless of this, the

splitting of the compounds under consideration by nucleosidase would be indicated by increasing amounts of iodine reduced due to the liberation of the aldehyde group. Our observations with nucleosidase are listed in Table III, which gives the increment of iodine reduction after incubation.

It is evident that the nucleosidase preparation does not split adenine thiomethylpentoside and isoguanosine. The action on hypoxanthine thiomethylpentoside is very limited and perhaps insignificant. According to Kalckar (12), nucleosidase action is a phosphorolytic process, yielding the labile ribose-1-phosphate. Accordingly, we checked the iodometric



I, adenosine; II, guanosine; III, isoguanosine; and IV, adenine thiomethylpentoside.

results listed in Table III by determination of phosphate, using Lowry's method (21). Only the splitting of guanosine was accompanied by phosphate uptake.

#### DISCUSSION

The relationship of the purine nucleosides used in our experiments may be seen from their formulas. It is known that guanosine is resistant toward adenosine deaminase (22). Nucleosides other than adenosine with the amino group in position 6 have not been examined earlier. The failure of deaminase to attack them is remarkable, because isoguanosine is different from adenosine only in that it has a hydroxy group instead of hydrogen in position 2, and adenine thiomethylpentoside differs in having a thiomethyl group in the carbohydrate moiety. The inability of deaminase to

split the latter compound was expected, because it resembles somewhat adenosine-5'-phosphoric acid, which is resistant to this enzyme. Not all modifications of the carbohydrate part, however, interfere; adenine desoxy-ribose is deaminated by adenosine deaminase (11).

Similar considerations hold for the results with nucleosidase. Earlier investigators (12, 23) found it capable of hydrolyzing only inosine and guanosine. The inertia of adenine thiomethylpentoside is analogous to that of adenosine, in which the amino group interferes with nucleosidase action. However, the failure of nucleosidase to split hypoxanthine thiomethylpentoside emphasizes the fact that the carbohydrate part also contributes to the specificity. A perfect fit (23) of the purine nucleus alone to the enzyme is not sufficient.

Our data amplify the information on specificity of enzymes concerned with nucleic acid metabolism. We agree with Dixon and Lemberg (23) in their criticism of the term "purine nucleosidase." It should be replaced by a more specific name.

#### SUMMARY

1. Experiments with adenosine deaminase from small intestine showed that 2-hydroxy-6-aminopurine-D-ribose (crotonoside, isoguanosine) and adenine thiomethylpentoside are not deaminated.

2. The same compounds are not split by nucleosidase from mammalian tissue.

3. The implications of these findings with respect to enzyme specificity are discussed.

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## METALLOPORPHYRINS

### VI. CYCLES OF CHANGES IN SYSTEMS CONTAINING HEME

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#### INTRODUCTION

An artificial system containing a metalloporphyrin and a substance that coordinates with it has several aspects that are of interest to the biochemist. It is partly analogous to several natural systems. It exhibits that reversible combination between diverse substances, many instances of which are being revealed among the biological catalysts and among other components of the body. The changes in a system containing an iron porphyrin involve exchanges of protons, electrons, and coordinating substance, thereby providing the opportunity to integrate the free energy changes of three distinct types of process—types of process that undoubtedly are integrated in the continuum of the still more complicated chemistry of the living organism.

For these reasons a precise description of such a system should serve as that of a model system. Unfortunately complexities and experimental difficulties have militated against that precision of measurement which is desirable in the definitive description of a model. Nevertheless, certain features having broad significance were outlined in the reports by Conant *et al.* (1, 2), by Hogness, Zscheile, Sidwell, and Barron (3), by Barron (4), and in previous papers of this series (5–9). What has been lacking is the completion of cycles of changes in one or another such system, cycles around which the experimentalist can lead the system by modifying conditions and components so as to resolve the system as a whole into part systems such as are represented in Figs. 2 and 10. This report is of an effort to find consistent relations by crisscross comparisons and completion of cycles of changes.

The importance of seeking consistency by this means is made the more evident by recollection of the following facts. (a) Limited solubilities of some components restrict attempts to obtain precise values for some of the equilibrium constants. (b) The involvement of several species places limitations upon the use of spectrophotometric data. (c) So high a concentration of a weakly coordinating base such as pyridine is required to approach

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saturation of a ferriporphyrin that the "thermodynamic environment" must be seriously altered and the unique features of weak coordination are difficult to define. (d) The accumulating evidences that the ferriporphyrins exist in aqueous solutions as micellae, that there are dimeric units within the micellae, and that in some instances the latter are split on addition of a coordinating substance suggest that ultimately a highly generalized treatment may have to be used. A roughing out of principal features seems advisable to show what simplifications are permissible.

For the above reasons it is important to state the policy followed in the analyses of the experimental data here reported. Whenever practical we have used those objective methods of analyzing data given in Paper I of the series and with the simplest postulates that are consistent with the data. The conclusions regarding one set of equilibria are accepted tentatively if consistent with related data. Obviously the conclusions drawn are not final; they serve to outline gross features of the system as a whole and to define those problems that will require more penetrating attacks to develop detail.

The original data are too voluminous to publish; so that it is impracticable to furnish all the details necessary for recalculations with assumptions other than those indicated by the graphic, mathematical, and other objective methods of analysis outlined in Paper I (5). This is not such a great loss as may appear, for our experience has been that each fresh attack usually reveals some new detail of these complicated systems. The severe condensation which has seemed advisable leaves sections of this paper almost cryptographic and about as attractive as little brush heaps in a clearing. The colonnades of a forest begin to appear in the summary.

A great deal of space is saved by referring to Paper I of this series (5) for the proofs of mathematical relations. The propositions given in Paper I will be cited by roman numerals.

The techniques and standards used were essentially those described in previous papers of this series (5-9). All measurements were at 30°.

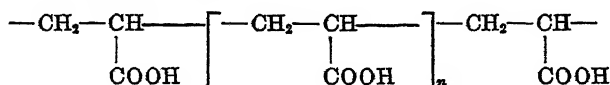
The evidence of aggregation to be presented raises the question of how it is that some of the data can be treated as if the molecules were completely dispersed. Also the evidence of dimerization of certain components presents the problem of dealing with dimers in various ways. Because neither of these aspects was treated adequately in Paper I, brief comment is appropriate here.

The problem of the acid with 2 or more removable protons had been treated in terms of the experimentally determined ionization constants when Adams (10) reopened the problem. He noted that structure may determine the first step in the ionization of  $\text{HR}_1\text{R}_2\text{H}$ , so that the ion  $\text{R}_1\text{-R}_2\text{H}$  rather than the ion  $\text{HR}_1\text{R}_2^-$  predominates, but that a general treatment re-

quires the assignment of *two formal* ionization constants for each of the first and second steps. The two constants for *each* step will be equal if the groups to which they pertain are the same and are at equivalent positions. Likewise they will be the same if the groups are parts of non-interacting molecules in a micella, and are equivalent. Subsequent treatments by Simms (11), by Weber (12), by von Muralt (13), by Hill (14), and by others have shown the mathematical relations between the formal constants and the experimental constants for an acid with  $n$  removable protons. The principles should apply not only to addition and loss of protons but also to addition and loss of electrons and addition and loss of coordinating substances.

In any case in which the addition or loss of 1 proton, electron, or molecule of coordinating substance affects the state of affairs at another group, or associated molecule, a subsequent change should be accompanied by a different free energy change, reflected in a different equilibrium constant. But if spatial or other factors interfere with transmission of the first effect, the subsequent change should not be accompanied by a significantly different free energy change. It then will appear that a molecule with  $n$  identical groups, or an aggregate of  $n$  identical molecules, will behave as would  $n$  simple molecules, with respect to a titration curve.

An *approach* to this is exhibited by those acidic groups of a protein which are identical; see for example Cannan, Kibrick, and Palmer (15). The titration curve of polyacrylic acid



might be expected to depart significantly from the form of the titration curve of a simple acid because the carboxyl groups are sufficiently close for the charge of 1 anion to affect the loss of protons from adjacent groups. A significant departure was found by Kern (16), but the important point that bears upon the present subject was Kern's finding that essentially the same titration curve was obtained whether the number of carboxyl groups was 50 or 340.

If, as in some of the cases to be presented, the titration curve conforms to one or another of the simpler forms, it may be assumed that interaction between the units of a large micella is so weak as to be negligible.

Some aspects of equilibria involving dimers may be treated in terms analogous to those used by Adams (10) in his discussion of the acid furnishing 2 protons.

Let  $L$  represent proton, electron, or coordinating substance added to a dimeric metalloporphyrin  $MM$ .  $L$  may be called a ligand. What Hill (14)

calls the microscopic constants will be represented by  $k$ . The equilibrium equations expressed with neglect of activity coefficients are

$$\begin{aligned}\frac{[MM][L]}{[MML]} &= k_{21} & \frac{[MML][L]}{[LMML]} &= k_{11} \\ \frac{[MM][L]}{[LMM]} &= k_{22} & \frac{[LMM][L]}{[LMML]} &= k_{12}\end{aligned}\quad (1)$$

As shown by Adams

$$K_1 = k_{11} + k_{12} = \frac{([MML] + [LMM])[L]}{[LMML]} \quad (2)$$

$$K_2 = \frac{k_{21}k_{22}}{k_{21} + k_{22}} = \frac{[MM][L]}{[MML] + [LMM]} \quad (3)$$

The following theoretical cases will have to be considered.

*Case 1*—When there is no interaction between the parts,  $k_{21} = k_{22} = k_{11} = k_{12}$ . Then  $K_1 = 4K_2$  and also the titration curve will have the form identical with that for the case

$$\frac{[M][L]}{[ML]} = K \quad (4)$$

When  $\log [L]$  is plotted against  $\alpha = [ML]/S$  (where  $S = [ML] + [M]$ ), the curve will be called one of the *first order*.

*Case 2*—When  $K_1/K_2 = 0$  (i.e.  $k_{11} + k_{12} = K_1 = 0$ ), the intermediates do not form, the equilibrium is described by

$$\frac{[MM][L]^2}{[LMML]} = K \quad (5)$$

and when  $\log [L]$  is plotted against  $\alpha = [LMML]/S$  (where  $S = [LMML] + [MM]$ ), the curve will be called a curve of the *second order*. The curve will be steeper than in Case 1. See also Propositions XII and XIV (5).

*Case 3*—When  $K_1/K_2 > 4$ , there will be interaction between the parts of the complex, and the titration curve will show signs of stepwise change. The titration curve will be drawn out along the axis  $\log [L]$  more than in Case 1. When  $K_1$  is very much larger than  $K_2$ , the steps will be distinct, as in the alkalimetric titration of several acids, or the reductive titration of one or another compound in a region of pH in which the intermediate "semiquinone" is stable (see Michaelis and Schubert (17)). Each part of this stepped curve will approach the form for Case 1.

*Case 4*—When  $K_1/K_2 < 4$  and  $> 0$ , the intermediates are of low stability

and the titration curve plotted with  $\log [L]$  will be less steep than a curve of the second order but steeper than one of the first order.

Whereas potentiometric measurements have to do more directly with the ligand, as proton or electron, spectrophotometric measurements are concerned more directly with the different species that acquire, or lose, protons, or electrons, or coordinating substance.

Let  $S = [MM] + ([LMM] + [MML]) + [LMML]$ . If we assume no optical difference between  $LMM$  and  $MML$  and no absorption by free  $L$ , the optical density,  $D$ , of a solution in a 1 cm. cuvette will be

$$D = \epsilon_0[MM] + \epsilon_1([LMM] + [MML]) + \epsilon_2[LMML]$$

When only  $MM$  is present,  $D_0 = \epsilon_0 S$ . When only  $LMM$  and  $MML$  are present,  $D_1 = \epsilon_1 S$ . When only  $LMML$  is present,  $D_2 = \epsilon_2 S$ . Hence

$$D = \frac{D_0 K_1 K_2 + D_1 K_1 [L] + D_2 [L]^2}{K_1 K_2 + K_1 [L] + [L]^2} \quad (6)$$

If there is no intermediate recognizable,

$$D = \frac{D_0 K + D_2 [L]^2}{K + [L]^2}$$

and if we define the degree of transformation by  $\alpha = [LMML]/S$ ,

$$\frac{D - D_0}{D_2 - D_0} = \frac{[L]^2}{K + [L]^2} \quad (7)$$

A plot of  $\alpha$  against  $\log [L]$  will give a symmetrical curve of the second order.

If the curve approaches the form of a curve of the second order but is slightly drawn out, it is presumptive evidence of the formation of an intermediate to a slight extent. It may be impracticable to determine  $D_1$ . But the presumption that the intermediate forms to only a small extent permits one to assign an arbitrary value to  $D_1$  without serious error. Then curve fitting can be accomplished with equation (6).

We shall have to deal with cases in which it appears that the addition of a ligand to a dimer is accompanied by a splitting of the dimer. For example, neglect possible intermediates and assume

$$\frac{[MM][L]^4}{[LML]^2} = K$$

Let  $S = 2[MM] + [LML]$  and let  $\alpha = [LML]/S$ . Then

$$\frac{S(1 - \alpha)}{2\alpha^2 S^2} = \frac{K}{[L]^4}$$

When  $[L]$  is kept constant and  $S$  is varied (the test of dilution),

$$\frac{d \log S(1 - \alpha)}{d \log \alpha S} = 2 \quad (8)$$

#### MATERIALS USED

The pyridine used was of "analytical reagent" grade. It was redistilled just prior to use and the middle fraction was taken.

Cyanide solutions were made with c.p. KCN from freshly opened bottles and were standardized with standard silver nitrate solutions.

The chloride of ferriprotoporphyrin IX (hemin chloride) was prepared by two methods. The first is described in Paper II of this series (6). The principal features are the use of well washed red blood cells and two or more recrystallizations by adding a chloroform solution of the quinine complex to hot glacial acetic acid. The assumption is that such recrystallization rids a preparation of material carried down in the first treatment with glacial acetic acid. The second method is a modification by Drabkin and Austin (18) of the procedure of Nencki and Zaleski (19). Well washed red cells are used and instead of recrystallization the original crystals are washed repeatedly.

We shall designate preparations made with the quinine treatment by Q and those made by the second method by D-A.

We should like to report absorption coefficients that characterize hemin chloride and its complexes. It is pertinent to give reasons for not doing so.

During his residence in this laboratory as Guggenheim Fellow Dr. David Drabkin made extensive spectrophotometric studies of several samples prepared by the two methods. We thank him for permission to comment briefly on a few of the results.

Q crystals were more "corroded" and the angles between faces and of extinction differed appreciably from those of D-A crystals. Solutions of Q preparations gave consistently lower absorption coefficients at certain wavelengths when converted to several of the base complexes. For example, several Q preparations when converted to the dicyanide ferriprotoporphyrin gave, at 545  $m\mu$ , the average absorption coefficient of 10.31, while under the same conditions D-A samples gave an average of 11.13, calculated for 1 mm and 1 cm. Comparable differences were found for the dipyrindine complexes.

These brief remarks do not cover all the differences noted by Drabkin. We need not give further details now because in the several cases in which samples of both types were used the same equilibrium constants were obtained within the limits set by our experimental error.

The important and still unresolved problem of characterizing ferriproto-

porphyrin by absorption coefficients of its complexes proved less disturbing in studies of the type here reported than have slow changes of absorption with time. Realizing that such changes do occur we have taken care to allow what we judged to be a reasonable time for the establishment of equilibrium

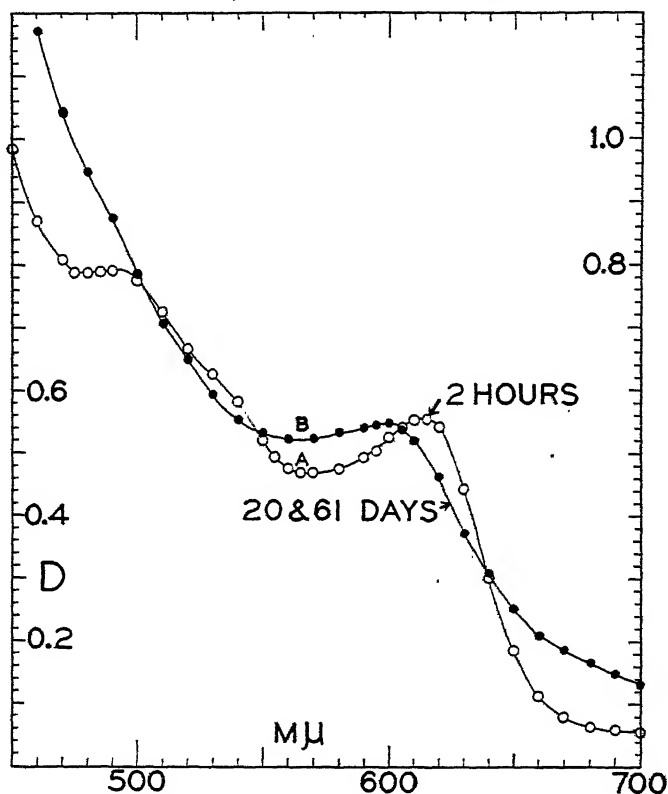


Fig. 1. Change with time of the optical density of an approximately 0.1 mM solution of ferritoporphyrin in phosphate buffer of about pH 11.3. Ordinate, density as observed with a Beckman spectrophotometer. Curve A observed immediately after preparation of solution; Curve B observed 20 days later and duplicated within the experimental error at the 61st day.

librium and to complete the measurements of a series of solutions within a short period thereafter. The subject, however, deserves more systematic study.

There are suggestions in the literature that solutions of ferritoporphyrin are unstable. In considering the following experiment one should realize that the conditions are not those which Lemberg (20) found favorable to the formation of "verdo hemes." Curve A of Fig. 1 shows the absorption

curve of an alkaline solution of ferriprotoporphyrin measured rapidly with a Beckman instrument *immediately* after preparation. Curve B pertains to the same solution 20 days later. Within the time interval intermediate points were observed, but with the exception of absorptions at the higher and lower wave-lengths there was practically no change within the time we usually allowed for our equilibrium measurements. After 61 days Curve B was duplicated within the experimental error. Comparable changes were observed with a solution preserved under hydrogen and comparable changes were observed with different alkaline buffer solutions. In the latter cases the contours of the absorption curves differed significantly.

In view of the findings regarding states of aggregation, discussed in the next section, it seems not out of place to make the following suggestion for the adequate testing of which we do not have the means. It is suggested that, while decomposition is not excluded, the changes may be due principally to changes in the distribution of particle sizes. Supporting this suggestion are the following considerations. The rolling contour of the absorption curve is similar to that of a mixture. The change of contour with change of buffer has a counterpart in the change of state of aggregation with buffer. The change with time reminds one of the change of diffusion coefficient with time. If the change with time were due to decomposition, it reasonably might be expected to progress continuously, whereas the same absorption curve was observed at the 20th and 61st days.

#### STATES OF AGGREGATION

Scattered through the literature are citations of evidence that heme in aqueous solution is far from being molecularly dispersed. Zeile and Reuter (21), in an article on cytochrome *c*, reported the diffusion coefficient of heme in  $M/15$   $Na_2HPO_4$  to be 0.0399 sq. cm. per day at  $0^\circ$ . From this they estimated a particle weight of approximately 50,000. Haurowitz (22), in an article on hemoglobin, reported that heme in 0.1  $M$   $NaOH$  at  $15^\circ$  showed a declining rate of diffusion with time. He estimated that during the first 0.29 day the average particle weight was 145,000 and over 4,000,000 at the end of 4 days of diffusion. He also noted a decline of apparent particle weight on addition of cyanide: 1900 at 0.75 day and 2300 at 5.7 days of diffusion. Gralén (23), in his study of the splitting of hemoglobin by acids, reported sedimentation constants for heme in alkaline solution,  $s_{20}$  varying from 2.2 to 3.6, indicating that the system is polydisperse. According to his data the particle weights vary from about 30,000 to 60,000.

We have assembled our data on diffusion in Table I. These data were obtained by the method of Northrop and Anson (24).

The cells with sintered glass membranes were standardized with 2  $M$  sodium chloride solution, as suggested by Anson and Northrop (25). When

a metalloporphyrin was used, the outer solution was made identical with the inner solution with respect to the components other than the salt of the

TABLE I  
*Diffusion of Metalloporphyrins*

Northrop and Anson method; all solutions approximately  $1 \times 10^{-3}$  molal;  $t = 30^\circ$ ; density of ferriprotoporphyrin chloride 1.37 (pycnometer); viscosity of buffer solutions assumed equal to that of water; mol. wt. of

$[\text{C}_{22}\text{H}_{22}(\text{C}_2\text{H}_4\text{COO})_2\text{N}_4\text{Fe}(\text{H}_2\text{O})(\text{OH})]^- \cong 649$  (additional hydration neglected).

Solution	<i>D</i>	Mol. wt., Stokes' law
Ferriprotoporphyrin		
	<i>sq. cm. per day</i>	
0.02 N NaOH	0.258	2,700
0.02 " "	0.246	3,100
0.02 " KOH	0.243	3,300
0.02 " NaOH + 0.08 M NaCl	0.137	18,000
0.02 " KOH + 0.08 " KCl	0.134	19,000
Borate buffer, pH 9.099*	0.110	35,000
" " " 9.212	0.102	44,000
Glycine " " 9.600	0.134	19,000
Phosphate buffer, pH 9.510	0.107	38,000
" " " 11.324	0.0784	98,000
" " " 11.388	0.0804	90,000
" " " 12.052	0.0916	60,000
Cyanide ferriprotoporphyrin		
0.02 N NaOH + 0.1 N NaCN	0.302	1,700
Phosphate buffer + 0.1 N KCN, pH 11.0	0.314	1,500
Ferrimesoporphyrin		
Phosphate buffer, pH 9.091	0.262	2,600
Pyridine ferriprotoporphyrin (viscosity of pyridine-water mixtures at $30^\circ = 1.49$ relative to water)		
Phosphate buffer, pH 10.59	0.150	9,300
" " " 7.83	0.1993	4,000

\* After standing 24 hours in 0.02 N NaOH to determine whether irreversible depolymerization occurred in the alkaline solution.

metalloporphyrin and the concentration of these other electrolytes greatly predominated (see McBain and Dawson (26) and McBain, Dawson, and Barker (27)). The outer fluid was changed until a stable concentration



gradient was established. To eliminate vibration the cells were placed in an air thermostat on a massive platform unconnected with the walls of the thermostat and its gently rotated fan.

The results given in Table I were calculated from averages of constant diffusion rates for two or more cells. From the diffusion coefficients, expressed in sq. cm. per day, the molecular weights can be estimated with Stokes' law only on the assumptions that the particles are of uniform size, have a spherical shape, and are uncharged. Consequently the significance to be attached to the estimations shown in Table I is only that the molecules of ferriprotoporphyrin in alkaline solutions are in large aggregates or micellae. The smallest particle size appears to be in 0.02 M alkali. Increase of particle size is occasioned by addition of chloride. Noticeable differences are found in borate, phosphate, and glycine buffer solutions.

The ratio  $D_{30}/D_0 = 2.48$ . Hence Zeile and Reuter's figure at 0° would be 0.099 at 30°, which agrees fairly well with our 0.107 for the comparable phosphate buffer.

The differences among diffusion coefficients for ferriprotoporphyrin are so great as to indicate that the forces holding the units in aggregation are very weak and are altered merely by small changes of electrolytes. If so, it is not surprising that these units may be treated as dispersed when the electrometric and other data on equilibria are considered.

Previously acquired evidence, as well as that now presented, indicates that a unit within the micella is a dimer which is split on addition of  $\text{CN}^-$  and may or may not be split by pyridine, depending on pH. Therefore, it is of interest to note the apparently smaller sizes of the aggregates in the presence of cyanide and in the presence of pyridine, as indicated roughly by the diffusion coefficients. It should be noted, however, that the minimal molecular weight of the ion  $[\text{C}_{28}\text{H}_{22}(\text{C}_2\text{H}_4\text{COO})_2(\text{N})_4\text{Fe}(\text{CN})_2]^-$  is about 666 (hydration being neglected).

As a rough check of our results the comparative rates of sedimentation in an ultracentrifuge were studied. We thank Dr. Morris Rosenfeld and the Department of Pharmacology for cooperating in this study. Because there was no optical system for measuring sedimentation rates or equilibria, the solutions were compared with solutions containing egg albumin and hemoglobin. The rotor contained fourteen cells, each divided into two parts by a septum of supported filter paper. After an appropriate period of rotation the solutions in the compartments were analyzed. The comparisons are shown in Table II. The data are in substantial agreement with the diffusion coefficients in the following respects. There is a higher degree of dispersion in the dilute alkali than in the buffer solutions. At an ionic strength of 0.1 and at  $2.8 \times 10^{-4}$  M, ferriprotoporphyrin is aggregated to particles, the sizes of which are of the order of magnitude of

the size of egg albumin (mol. wt. 40,000 to 46,000). Doubtless in no case is the state of aggregation uniform.

Anson and Mirsky (28) found that a collodion membrane which retains hemoglobin also retains heme in a "slightly alkaline" solution (composition and pH not specified). In some crude experiments with tubes of collodion and Visking cellulose sausage casing we observed the following interesting relations. 100 ml. of a 0.6 mM solution of ferriprotoporphyrin in 0.02 M KOH lost only 2.6 per cent during 24 hours dialysis against 890 ml. of 0.02 M

TABLE II  
*Ultracentrifugation of Ferriprotoporphyrin*

Solution	Molar concentration $\times 10^4$	Per cent of original in upper chamber
Series 1. 7 hrs. at 1000 revolutions per sec.		
0.02 N NaOH	10	63.0
Borate buffer, pH 9.0	10	6.8
" " " 10.0	10	19.1
Phosphate buffer, pH 11.0	10	1.8
Series 2. 5 hrs. at 1000 revolutions per sec.; ionic strength = 0.1		
0.02 N KOH + 0.08 M KCl, pH 12.1	2.8	12.4
0.01 " " + 0.09 " " " 11.8	2.8	15.9
0.005 " " + 0.095 " " " 11.5	2.8	15.1
0.002 " " + 0.098 " " " 10.9	2.8	13.2
0.001 " " + 0.099 " " " 8.2	2.8	14.1
Phosphate buffer, pH 11.7	0.28	41.9
" " " 11.7	2.8	18.4
" " " 11.8	2.8	14.9
" " " 12.3	2.8	14.4
" " " 6.95	0.28	21.2
Hemoglobin, pH 7.0		4.0
Egg albumin, pH 7.0		17.7

KOH, and with four renewals of the outer solution only 9 per cent dialyzed in 1 week. As indicated by the diffusion measurements, the average particle size is larger in borate buffer. In rough agreement with this was our observation that a 0.6 mM solution of ferriprotoporphyrin in Clark and Lubs' borate buffer of pH 9.0 lost only 1.3 per cent to 940 ml. of dilute borax solution in 1 day and thereafter, with four changes of outer solution, only detectable but not precisely measurable amounts were lost during a week.

Comparable results were obtained with collodion bags made rather permeable by use of ethylene glycol according to the receipt of Pierce (29). Ob-

servations with collodion were carried out only during a day because of the slow deterioration of the collodion in the slightly alkaline solutions.

As shown by Hogness *et al.* and confirmed by us, spectrophotometric data on the effect of adding cyanide to ferriprotoporphyrin can be accounted for on the assumption that a dimer is split. The diffusion data suggest a large increase in dispersion. In conformity therewith is our observation that the cyanide complex of ferriprotoporphyrin passes very rapidly through Visking cellulose sausage casing and collodion membranes. In one experiment the same sausage casing that permitted this rapid passage subsequently held ferriprotoporphyrin in 0.02 M KOH with very little loss during dialysis over a period of 2 weeks.

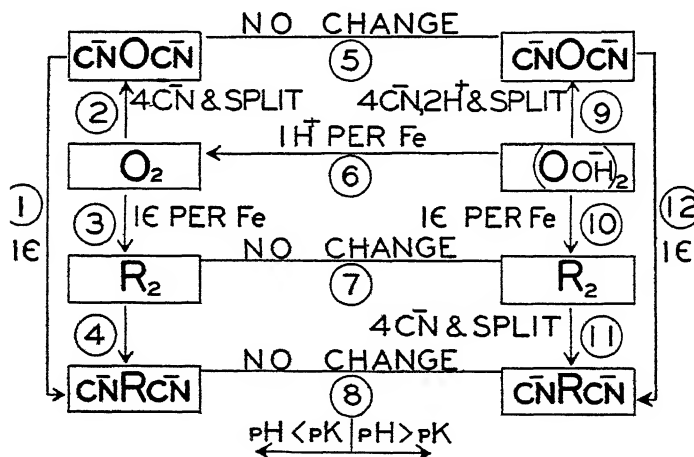


FIG. 2. Scheme of postulated relations, showing by numbers the processes, equilibrium states of which were studied. See the text, Sections I and II.

Spectrophotometric data also suggest that ferriprotoporphyrin in aqueous solution forms micellae. Comment on this will be made later.

#### A SCHEME TO AID SYSTEMATIC CORRELATIONS

A systematic treatment may be followed with the aid of Figs. 2 and 10. Substances in the upper two horizontal rows are postulated oxidants; those in the lower two rows are postulated reductants. Substances in the left-hand vertical column are postulated for solutions of relatively low pH; those in the right-hand column are postulated for solutions of relatively high pH. By relatively high and low pH we mean relative to the pK values, which are not the same for all systems.

That the carboxyl groups of the porphyrin are ionized in solutions of the pH range used may be inferred from previous findings and was demon-

strated by Porter (30) for the case of ferricoproporphyrin. Accordingly we may assume that the simplest ion of ferriprotoporphyrin IX is  $[\text{C}_{38}\text{H}_{22}(\text{C}_2\text{H}_4\text{COO})_2\text{N}_4\text{Fe}]^-$  which will be represented by **O**. The corresponding ion of ferroprotoporphyrin will be represented by **R**. As noted in Paper III (7), water molecules might be assumed to complete the coordination number, 6, of iron, when the 4 pyrrol nitrogens (to 2 of which there may be assigned a *formal* charge of 2-) and such other coordinating substance as may be specified do not suffice to complete that number. We have not resorted to this artistry, however, because we have no definite proof of the structural nature of the dimers and aggregates in the bonding of which the coordination shells, or water attached thereto, may play a part. Accordingly, and until there is occasion to consider coordinated water molecules, they will not be represented in formulas.

*I. Systems in Absence of Coordinating Substances Other Than Those  
Furnished by Aqueous Buffer Solutions*

*Process 10*<sup>1</sup> (See Fig. 2)—Conant *et al.* (1, 2) and Barron (4) reported some potentiometric measurements of the equilibrium states of this process but found uncertain potentials. Such was the experience in this laboratory until we added a mediator, when reproducible potentials were obtained. Indigo monosulfonate, or disulfonate, used in the proportion of about 4 per cent of the heme, served as mediator. The data were corrected for the amount of mediator reduced and then were analyzed by use of Propositions I, II, and XVI (see Paper I (5)). A typical set of results is shown in Fig. 3. Apparently  $n = 1$ , which may be interpreted as showing insignificant interaction between the iron atoms in an aggregate of the metalloporphyrin molecules. The form of the curve is that of Case 1 (p. 146). It would not necessarily preclude the postulation of dimeric oxidant and dimeric reductant provided the postulate did not extend to a bonding too intimately concerned with the iron. It precludes any difference in states of aggregation between oxidant and reductant that involves a significant free energy change.

*Process 6*—Within the range of pH 9 to 12.6, within the range of wavelengths used, and within the time of the experiment, no significant deviation from Beer's law was observed. No significant dilution effect was observed at wave-lengths from 500 to 660  $\text{m}\mu$  over the concentration range  $1.8 \times 10^{-4}$  to  $3.7 \times 10^{-4}$   $\text{M}$ .

The change of absorption in solutions of lower pH was investigated in an effort to estimate  $\text{pK}'_a$ . Two difficulties were encountered. In the first place even very dilute solutions are supersaturated at the lower pH

<sup>1</sup> The processes referred to by number in this and the following sections are those indicated by numbers in Figs. 2 and 10.

values; so that it was necessary to add an alkaline solution of the hemin chloride to the buffers. The very dilute solutions thus formed apparently were stable because the absorption of each remained constant for a day. Secondly, the changes of absorption with change of pH were small, about  $-\log T_1 = 0.3$  to  $-\log T_2 = 0.4$ . Thus the maximal deviation from the upper curve of Fig. 4 corresponds to an "error" of approximately 0.005 in optical density.

When the optical densities were analyzed to relate degree of ionization to pH, there appeared signs of asymmetry in the curve, rendering rectifica-

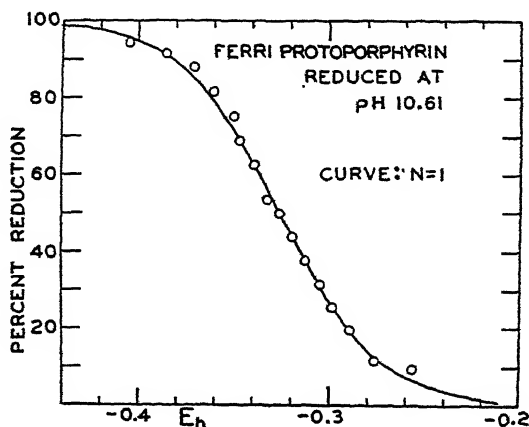


Fig. 3. Potentiometric test of Process 10. Reductive titration of ferriprotoporphyrin IX at constant pH. Curve, theoretical for  $n = 1$ . Reducing agent titanous tartrate. Mediator indigo monosulfonate. Total metalloporphyrin  $4.6 \times 10^{-4}$  M. pH of borate buffer 10.61. Ionic strength 0.1. Temperature 30°. Analysis of data by Proposition XVI.  $d = -0.166$  ml. Average  $E'_0 = -0.3257$ .

tion by Proposition XIV impractical. This suggested that a change in state of aggregation accompanies the change of pH, and accordingly an experiment was made to test the dilution effect. It happens that a fair curve fitting is obtained by assuming the process to be  $2O(OH^-) + 2H^+ \rightleftharpoons O_2 + 2H_2O$  and it is on this basis that the line in the lower part of Fig. 4 is drawn. This assumption, however, is inconsistent with much better evidence that the metalloporphyrin in alkaline solution is a dimer.

The case appears to be one in which final judgment must be based on the results of various approaches. A return to the species of the oxidant in acid solution by Process 2 will indicate that the dimeric form should be retained as specified in Fig. 2. Outstanding against this conclusion is the dilution effect exhibited by Fig. 4 but there does remain the possibility that the slight changes in absorption with change of pH, on which the calcula-

tions were based, may have been complicated by slight changes of the larger aggregates rather than of the dimer in these supersaturated solutions. Also no correction for scattering has been made and this is rather large.

Allowing for all this and the inability to define definitely the species, the order of magnitude of pH at which the "alkaline form" of ferriprotoporphyrin is half converted to the "acid form" has certainly been determined roughly, and we believe for the first time. The estimated value, pH 7.6,

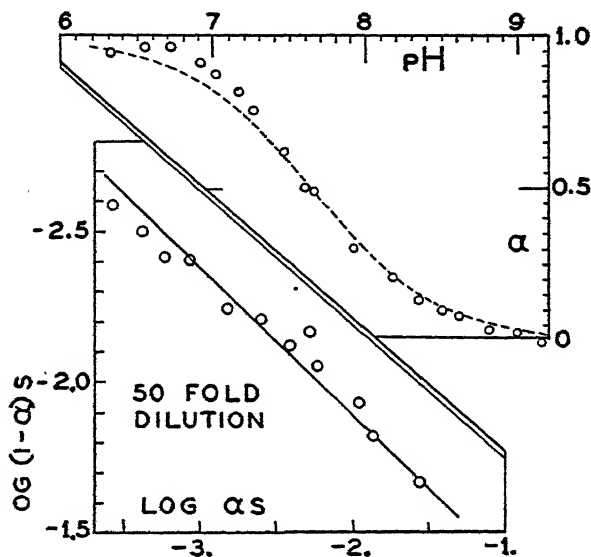


FIG. 4. Conflicting spectrophotometric tests of Process 6. Dotted upper curve, theoretical for a simple acid, or a dimer with no interaction between parts.  $\alpha = [\text{acid form}]/S$ ;  $S = \text{total metalloporphyrin} = 9.54 \times 10^{-5} \text{ M}$ . Lower curve, dilution effect; curve for  $2(\text{OOH}^-) + 2\text{H}^+ \rightleftharpoons \text{O}_2 + 2\text{H}_2\text{O}$ . See equations (30) to (33), Paper I. Slope for  $a/c = 2$ . See the text for other interpretation.

is reasonably close to 7.4 estimated indirectly, as will be shown later, and to 7.4 for ferricoproporphyrin estimated by Clark and Perkins (9).

It should be noted that this half transformation point pertains to a proton, or protons, added at the "coordination center" as indicated by a variety of evidences already set forth in previous papers.

If the relation is

$$\frac{[\text{O}_2(\text{OH}^-)_2][\text{H}^+]^2}{[\text{O}_2]} = K', \quad \text{p}K' = 2 \times 7.6 = 15.2$$

*Processes 6 and 7*—Both of these processes are involved when a fixed mixture of oxidant and reductant is submitted to changes in pH. In Fig. 5

are plotted the potentials at 50 per cent reduction obtained by individual titrations in solutions of the indicated pH values. In the alkaline region  $\Delta E_h/\Delta \text{pH} = -0.0601$  volt per unit of pH. According to Propositions X and XI this indicates that precisely one more hydroxyl group (or 1 less proton) is contained in the oxidant than in the reductant for *each* electron required to convert oxidant to reductant. It would not preclude  $n$  hydroxyls, or  $n$  protons, per  $n$  electrons. The "0.06 slope" is in agreement with the

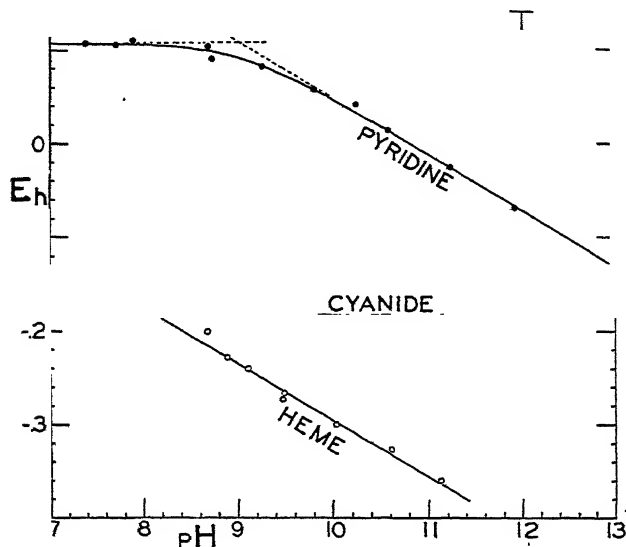


FIG. 5. Relation of pH to mid-points of reductive titration curves. Tests of Processes 6 and 7 combined, Processes 5 and 8 combined, and Processes V and VIII combined (see Fig. 10).  $t = 30^\circ$ . "Heme" represents half reduced ferriprotoporphyrin IX; total metalloporphyrin  $4.6 \times 10^{-4}$  M; intersection of dotted projections based on assumption that mid-point of Process 6 is at pH 7.4. "Cyanide," half reduced cyanide ferriprotoporphyrin IX; data of Barron;  $E'_0 = -0.183$ . "Pyridine," half reduced pyridine ferriprotoporphyrin IX; total metalloporphyrin  $1.0 \times 10^{-4}$  M; total pyridine 2.5 M; intersection of projections at pH = 9.04. See the comment on  $n$  in solutions of different pH.

trend of Barron's data, which were uncertain for the reason previously mentioned. We, like Davies (7), have not found those specific buffer effects reported by Barron. Our values for phosphate and borate buffers are very close to the linear relation. Barron's value for phosphate at pH 11.0, calculated to be  $-0.355$  volt, is in substantial agreement with our value of  $-0.353$  volt.

In solutions of lower pH the potentials drifted, probably because of the precipitation of components. For this reason it was impracticable to de-

termine  $pK'_2$  by finding the intersection of projections of two parts of an entire curve (Proposition XI).

For various reasons we have postponed exploration of solutions of ferriprotoporphyrin at extremely high pH. Our preliminary spectrophotometric data suggest either that increase of pH results in an increased dispersion, such as is indicated in Table I, or that there is a second ionization. We are informed by T. Harrison Davies<sup>2</sup> that his data on magnetic susceptibilities indicate a second ionization of ferriprotoporphyrin with  $pK'_2 = 12.45$ . This can be accounted for by the stripping of a proton from a coordinated water molecule.

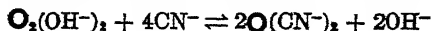
*Process 7*—Previous analyses of potentiometric data indicate no change of a reduced iron porphyrin with change of pH in that region of pH within which a hydroxyl group (or its equivalent) is associated with the oxidized metalloporphyrin. This we have confirmed in the case of ferroprotoporphyrin IX by finding identity of the spectra in the region pH 7 to 12. Clark and Perkins (9) report the same for ferrocoproporphyrin. Also we found no dilution effect in the concentration range  $1.83 \times 10^{-6}$  to  $3.66 \times 10^{-4}$  M.

Inasmuch as Processes 2, 4, 9, and 11 apparently involve the splitting of dimers, we have preserved the dimeric forms of oxidized and reduced metalloporphyrin at high and low pH, as indicated in Fig. 2.

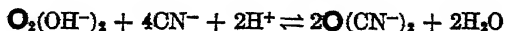
## II. Systems with Cyanide Ions Present

Processes 5 and 8 are involved when a fixed mixture of ferroprotoporphyrin and ferriprotoporphyrin, both saturated with cyanide, is placed in buffers of different pH values. The electrode potential does not vary with pH, as was shown by Barron (4) and confirmed by Davies (7), Vestling (8), and Porter (30) for other iron porphyrins. This means that cyanide at higher concentrations has displaced  $OH^-$  or its equivalent from the "coordination center" of any ferriporphyrin. Barron gives  $E'_0 = -0.183$  volt. See Fig. 5.

*Process 9*—Hogness *et al.* (3) obtained, by spectrophotometry, data that can be described with deductions drawn from the postulate



or

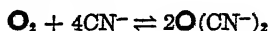


We have confirmed their results and have extended studies to solutions of pH values as low as 6.72.

<sup>2</sup> Private communication.



*Process 2*—The association curve at pH 6.72 conforms to the equilibrium equation determined by the process



A comparison between our data at pH 6.72 and 12.06 is given in Fig. 6. Note that the abscissa is  $\log [\text{KCN used}]$  and not  $\log [\text{CN}^-]$ .

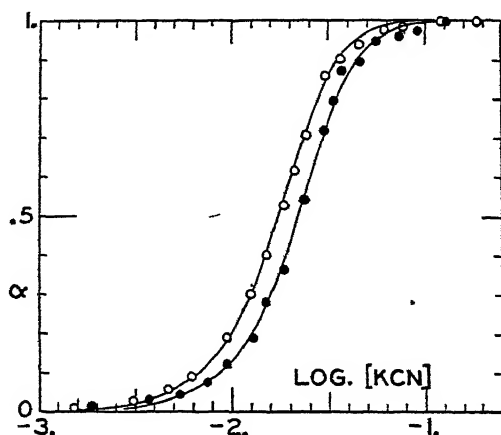


Fig. 6. Spectrophotometric tests of Processes 9 and 2. Association curves.  $m\mu$  545. Theoretical curves drawn for

$$\frac{[(\text{O}(\text{OH})_2)[\text{CN}^-]^4]}{[\text{O}(\text{CN})_2]^2} = \frac{K}{[\text{H}^+]^2} \quad K'$$

at constant pH. Ordinate,  $\alpha = [\text{O}(\text{CN})_2]/S_0$ ; abscissa,  $\log [\text{total KCN}]$ .  $\circ$  at pH 12.16;  $S_0 = 5.76 \times 10^{-5} \text{ M}$ ; ionic strength 0.1; half transformation at  $\log [\text{KCN}] -1.76$ .  $\bullet$  at pH 6.75;  $S_0 = 5.95 \times 10^{-5} \text{ M}$ ; ionic strength 0.1; half transformation at  $\log [\text{KCN}] = -1.65$ .

### *Apparent Ionization Constant of HCN*

For part of the following development there is needed the apparent ionization constant of HCN as found under the conditions of the experiments.

The apparent constant was determined with mixtures of 0.2 M KCN and 0.2 M HCl, each solution being diluted to ionic strength 0.1. The glass electrode was used. The potentials observed with these mixtures and with buffer solutions prepared with potassium salts were plotted and the pH of each cyanide solution was interpolated between those of the buffer solutions as determined with the hydrogen electrode. The titration curve was rectified by Proposition XVI and yielded the apparent ionization exponent 9.2 at 30°. This value was used in calculating  $[\text{CN}^-]$  for the first of the following tests. Britton and Robinson (31) give  $\text{p}K' = 9.32$  at 18°.

Processes 2 and 9 are involved in solutions of intermediate pH.

It is obvious that the cyanide ion competes well against hydroxyl ion for position in the coordination center, even at high pH. It is interesting to observe certain consequences of the progressive relief from the necessity of this competition as the hydroxyl ion concentration is reduced.

The equilibrium equation for Process 9 may be expressed by equation (9) according to Hogness *et al.* and with neglect of stepwise association.

$$\frac{[(\text{OOH}^-)_2][\text{CN}^-]^4[\text{H}^+]^2}{[\text{CN}^-\text{OCN}^-]^2} = K \quad (9)$$

Define the concentration of *uncombined* metalloporphyrin by

$$S_u = 2[(\text{OOH}^-)_2] + 2[\text{O}_2] \quad (10)$$

Introduce the acid equilibrium equation

$$\frac{[(\text{OOH}^-)_2][\text{H}^+]^2}{[\text{O}_2]} = K'_2 \quad (11)$$

Whence

$$\frac{S_u[\text{CN}^-]^4}{[\text{CN}^-\text{OCN}^-]^2} = \frac{2K}{K'_2} \left( \frac{K'_2 + [\text{H}^+]^2}{[\text{H}^+]^2} \right) \quad (12)$$

When the value of  $S_u/[\text{CN}^-\text{OCN}^-]$  is a constant,  $A$ , we have the following relations between pH and  $[\text{CN}^-]$ . When  $[\text{H}^+]^2 < K'_2$ , or  $\text{pH} \gg \text{p}K'_2$ ,

$$\log A + 4 \log [\text{CN}^-] = 2\text{pH} + \log 2K \quad (13)$$

$$\frac{\Delta \log [\text{CN}^-]}{\Delta \text{pH}} = \frac{1}{2} \quad (14)$$

When  $[\text{H}^+]^2 \gg K'_2$ , or  $\text{pH} < \text{p}K'_2$ ,

$$\log A + 4 \log [\text{CN}^-] = \text{p}K'_2 + \log 2K \quad (15)$$

$$\frac{\Delta \log [\text{CN}^-]}{\Delta \text{pH}} = 0 \quad (16)$$

In Fig. 7 are plotted, in the upper curve, data relating  $\log [\text{CN}^-]$  and pH for the case in which the metalloporphyrin was half transformed to the cyanide metalloporphyrin. These data were obtained by using the half transformation points of association curves. For high values of pH relation (14) is obeyed, showing that progressively lower concentrations of  $\text{CN}^-$  are required as pH declines. Although the points are somewhat scattered for the lower pH values, it is clear that at the lower pH values relation (16) is obeyed when the metalloporphyrin contains no hydroxyl against which  $\text{CN}^-$  must compete.

The intersection of the projections of the two sections of the curve will occur when, by relations (13) and (15),  $\text{pH} = \text{pK}'_a/2$ . If the metalloporphyrin is considered a univalent acid, the intersection would be at  $\text{pH} = \text{pK}'_a$ . To be general we may call the intersection a half transformation point. It is found to be 7.4. With due consideration of the indirectness of this method, which involves the assumption of an ionization constant

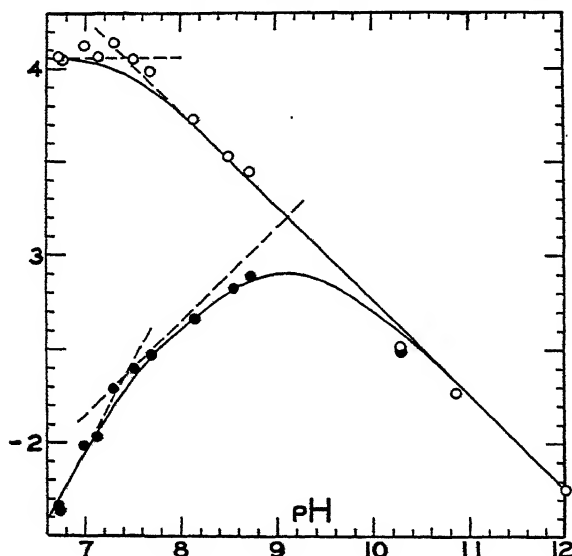


Fig. 7. O, relation of pH (abscissa) to  $\log [\text{CN}^-]$  (ordinate) required to maintain equimolecular mixture of ferriprotoporphyrin and dicyanide ferriprotoporphyrin. Intersection of projections at pH 7.4, the point of half transformation of alkaline form to acid form of ferriprotoporphyrin. ●, relation of pH to  $\log ([\text{CN}^-] + [\text{HCN}]) = \log S_c$ .  $S_c$  such as to maintain equimolecular mixture of ferriprotoporphyrin and its cyanide complex. Slopes of main parts of curve: at high pH,  $\Delta \log S_c / \Delta \text{pH} = \frac{1}{2}$ , at intermediate pH,  $\Delta \log S_c / \Delta \text{pH} = -\frac{1}{2}$ , and at low pH,  $\Delta \log S_c / \Delta \text{pH} = -1$ . Intersections, pH = 7.4 = point at which metalloporphyrin is half transformed from  $\text{O}_2$  to  $\text{O}_2(\text{OH})_2$ ; pH = 9.1 = point at which HCN is half transformed to  $\text{CN}^-$  (compare with 9.2 measured directly).

for HCN with which  $[\text{CN}^-]$  was calculated, the numerical value of this half transformation point is in substantial agreement with 7.6 estimated by the more direct method, which, it has been noted, was not highly precise.

The above development was predicated tacitly on the assumption that  $\text{CN}^-$  coordinates with the metalloporphyrin and that HCN does not. This must now be tested.

$$\text{Let } S_c = [\text{CN}^-] + [\text{HCN}] \quad (17)$$

$$K'_{cs} = \frac{[\text{CN}^-][\text{H}^+]}{[\text{HCN}]} \quad (18)$$

Substitute equations (17) and (18) in equation (12) and from the resulting equation find the following for a fixed ratio of uncombined metalloporphyrin to cyanide metalloporphyrin.

$$\text{When } \text{pH} \gg \text{pK}'_a \text{ and } \text{pH} \gg \text{pK}'_{ac}, \frac{\Delta \log S_o}{\Delta \text{pH}} = \frac{1}{2} \quad (19)$$

$$\text{When } \text{pH} \gg \text{pK}'_a \text{ and } \text{pH} \ll \text{pK}'_{ac}, \frac{\Delta \log S_o}{\Delta \text{pH}} = -\frac{1}{2} \quad (20)$$

$$\text{When } \text{pH} \ll \text{pK}'_a \text{ and } \text{pH} \ll \text{pK}'_{ac}, \frac{\Delta \log S_o}{\Delta \text{pH}} = -1 \quad (21)$$

Because values of  $\text{pK}'_a$  and  $\text{pK}'_{ac}$  are not sufficiently different to maintain the intermediate linear slopes over a large range of pH, it is necessary to make some trial calculations to arrive at a theoretical curve which will describe the data fairly well. Once this is accomplished there can be calculated, as if independent, the three lines which will intersect at the half transformation point of the metalloporphyrin ( $\text{pK}'_a$ , treated as that of a simple acid) and at the pH corresponding to the ionization exponent of HCN. These intersections, illustrated in Fig. 7, are as follows: half transformation point of ferriprotoporphyrin as an acid = 7.4; half transformation point of HCN = 9.1.

The second independent evaluations of ionization exponents, or rather of half transformation points, have been reached with only the following assumptions: (a) that by use of spectrophotometric data the metalloporphyrin has been maintained half transformed to the cyanide complex; (b) that only  $\text{CN}^-$ , and not HCN, coordinates with the metalloporphyrin. The check is clear evidence that  $\text{CN}^-$ , and not HCN, is involved in the particular process considered.

Here we may reemphasize an interesting relation made apparent by projection of previous potentiometric data into the region of pH 7.0. By applying Proposition III to the data for association of  $\text{CN}^-$  with ferro- and ferrimetalloporphyrins in mixture, it is shown that  $\text{CN}^-$  combines more "tightly" with the ferroporphyrin than with the ferriporphyrin in *alkaline* solution. As pH is lowered,  $\text{CN}^-$  has less competition against  $\text{OH}^-$  in the ferrimetalloporphyrin. The curve relating potential to pH for the case when both the reduced and oxidized metalloporphyrins are saturated with  $\text{CN}^-$  can be projected to cross the curve for the system without cyanide, as shown in Fig. 5. Thus, at low pH, at which  $\text{CN}^-$  is relieved from competing with  $\text{OH}^-$ ,  $\text{CN}^-$  combines more "tightly" with the oxidized than with the reduced metalloporphyrin. But as already noted, only  $\text{CN}^-$  combines; so that at pH much lower than the  $\text{pK}'_{ac}$  of HCN a high concentration of total cyanide is necessary.

*Process 8*—Within the range of wave-length explored (450 to 710  $m\mu$ ) no change in the visible spectrum with change of pH (7.4 to 12.6) was found. This is in agreement with a deduction to be drawn from the potentiometric data of Barron (4) and Davies (7) for the combined Processes 5 and 8. At high concentration of  $CN^-$ , sufficient to displace  $OH^-$  from the oxidant

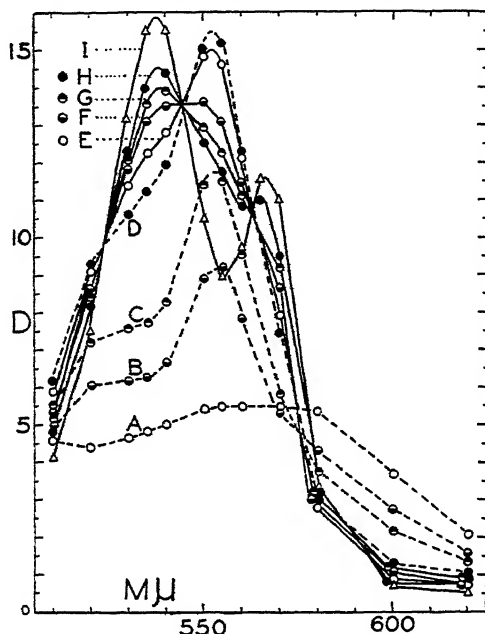


Fig. 8. Spectrophotometric test of Process 11. Absorption curves of ferroprotoporphyrin alone and in the presence of cyanide at different concentrations. Abscissa, wave-length in  $m\mu$ ; ordinate,  $-\log T$  for 1 mm total metalloporphyrin, and 1 cm.

Curve	Symbol	Total cyanide	Curve	Symbol	Total cyanide
A	○	0	E	○	$3.68 \times 10^{-4} M$
B	●	$9.20 \times 10^{-5} M$	F	●	$5.52 \times 10^{-4} "$
C	○	$1.47 \times 10^{-4} "$	G	●	$7.36 \times 10^{-4} "$
D	●	$2.76 \times 10^{-4} "$	H	●	$9.20 \times 10^{-4} "$
			I	△	$5.52 \times 10^{-3} "$

$O_2(OH^-)_2$ , a fixed ratio of total oxidant to total reductant gives an electrode potential invariant with pH; see Fig. 5.

*Process 11*—Anson and Mirsky (32), using a spectroscopic method, found evidence of stepwise combination of cyanide with ferroprotoporphyrin. They and Hill (33) estimated that 1 cyanide ion is involved at each step. That 2 cyanide ions associate with the reduced metalloporphyrin per 1 iron atom was shown by Davies' (7) potentiometric measurements, which gave  $q = r = 2$  (by Propositions IV and VI).

Inasmuch as our own studies of this process have failed to yield precise values of the equilibrium constants, only a sketch of our results is given here. In Fig. 8 are shown the absorption curves of ferroprotoporphyrin without and with various proportions of cyanide. A distinct shift of the peak of absorption is evident. Presumably this indicates a change of species. Because a simple treatment of spectrophotometric data is limited to the case of two absorbing species we have proceeded as follows.

At  $545\text{ m}\mu$ , where there is an isosbestic point, and at  $600\text{ m}\mu$  it appears that the monocyanide complex and the dicyanide complex have the same

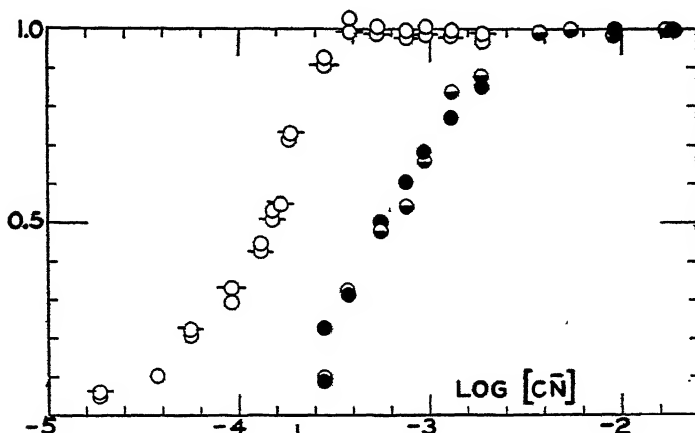


Fig. 9. Crude spectrophotometric test of Process 11. Relation of  $\log [\text{CN}^-]$  to degree (ordinate) of conversion of ferroprotoporphyrin to monocyanide and dicyanide ferroprotoporphyrin. Placement of points calculated after arbitrary resolution of spectrophotometric data.  $\circ$ , first step to monocyanide by data at  $545\text{ m}\mu$ ;  $\odot$ , first step to monocyanide by data at  $600\text{ m}\mu$ ;  $\bullet$ , second step to dicyanide by data at  $555\text{ m}\mu$ ;  $\bullet$ , second step to dicyanide by data at  $538\text{ m}\mu$ . Total metalloprophyrin  $6.9 \times 10^{-5}\text{ M}$ .  $t = 30^\circ$ .

absorption coefficient. Accordingly the optical densities at these wavelengths were used to obtain the relation between  $\log [\text{CN}^-]$  and the ratio  $\frac{[\text{RCN}^-] + [\text{CN}^- \text{ R CN}^-]}{S}$  where  $S$  is the sum of the concentrations of all

forms. To estimate the absorption curve for the monocyanide complex the optical densities on the absorption curve at  $\log [\text{CN}^-] = -3.735$ , where  $\alpha = 0.72$ , were multiplied by  $1.0/0.72$ . Using this curve for the pure monocyanide as standard we could estimate the proportions of the dicyanide at various values of  $\log [\text{CN}^-]$ . The calculated values are plotted in Fig. 9.

Obviously the scatter of the points is such as to preclude any definitive calculation of constants or of the precise forms of the curves. The curves

are somewhat steeper than expected on any simple basis. A similar situation will be noted in the discussion of the association of pyridine with ferroprotoporphyrin.

The half transformation points apparently are at  $\log [\text{KCN}] = -3.8$  (first step) and  $-3.24$  (second step).

*Check by Combination of Constants*—Because there is no change in Process 5 (see Fig. 2), the equilibrium constant for Process 9 should equal the product of the equilibrium constants for Processes 6 and 2 when the equations are written as follows:

$$K_9 = \frac{[(\text{OOH}^-)_2][\text{CN}^-]^4[\text{H}^+]^2}{[\text{CN}-\text{O}^-\text{CN}]^2} = \frac{[(\text{OOH}^-)_2][\text{H}^+]^2}{[\text{O}_2]} \times \frac{[\text{O}_2][\text{CN}^-]^4}{[\text{CN}-\text{O}^-\text{CN}]^2} = K_6 \times K_2 \quad (22)$$

When  $[(\text{OOH}^-)_2]/[\text{CN}-\text{O}^-\text{CN}] = 1$ ,  $4 \log [\text{CN}^-] - \log S - 2 \text{ pH} = \log K_9$ .

From the data in the legend of Fig. 6 we have

$$4(-1.76) - (-4.24) - 2(12.16) = -27.12 = \log K_9$$

When  $[(\text{OOH}^-)_2]/[\text{O}_2] = 1$ ,

$$\begin{aligned} -2 \text{ pH} &= \log K_6 \\ -2(7.6) &= \log K_6 = -15.2, \text{ or } -2(7.4) = \log K_6 = -14.8 \end{aligned}$$

depending on which estimate of the half transformation point is used. We shall use the average,  $-15.0$ .

When  $[\text{O}_2]/[\text{CN}-\text{O}^-\text{CN}] = 1$ ,  $4 \log [\text{CN}^-] - \log S = \log K_2$ .

$\log S = -4.23$ ; see the legend of Fig. 6. The half transformation point was at  $\log [\text{total cyanide}] = -1.65$ . If  $\text{pK}'_{ac}$  for HCN is 9.2, the value of  $\log [\text{CN}^-]$  at pH 6.75 is about  $-4.097$ . Hence  $4(-4.097) - (-4.23) = -12.16 = \log K_2$ . Then  $\log K_9 = -27.12$  should equal  $-15.0 - 12.16 = -27.16 = \log K_6 + \log K_2$ . The agreement is better than the agreement between the estimates of  $\log K_6$ . It should be noted that no consideration has been given to certain matters which should be considered in a precise treatment. Activity coefficients have been neglected. Measurements, however, were at comparable ionic strengths. Possibly consideration will have to be given to the participation of water. Apparently the free energy of association of the molecules in micellae is of second order magnitude, or it has been automatically taken care of in the formulation of a split of a "dimeric unit."

### III. Systems with Pyridine

Previous studies of the coordination of pyridine with one or another metalloporphyrin have shown that a very high mole fraction of pyridine is required to approach saturation. The consequent large change of the

"thermodynamic environment" and dielectric constant would make it appear difficult to formulate data by any simple rules based on equations for ideal solutes. On the other hand, pyridine binds so weakly with metalloporphyrins and competes so poorly with  $\text{OH}^-$  for position at the coordination shell of a ferrimetallporphyrin that parts of the entire system are in marked contrast to comparable systems containing cyanide. The resulting unique features are so interesting that we were tempted to investigate the cycle of changes represented in Fig. 10 in order to see whether there would appear relations of consistency sufficient to justify the gross formulation of the several components. Furthermore pyridine offers an advantage over

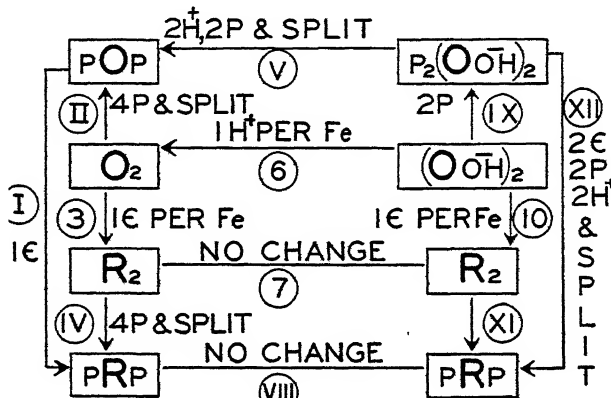


FIG. 10. Scheme showing by numbers the processes investigated. Compare with Fig. 2.

certain other coordinating substances in that its ionization constant is such that practically only the base is present within the range of pH used. The complexities incident to dealing with more than one species of the coordinating substance are avoided.

In Fig. 10 Processes 3, 6, 7, and 10 have already been discussed. They are represented in Fig. 2.

*Process I*—Reductive titrations at relatively low pH, and in the presence of high, constant concentrations of total pyridine, gave symmetrical titration curves. The data for one case are shown in Table III. Rectification by means of Proposition XVI indicates that  $n = 1$ . At constant pH and constant per cent reduction the potentials varied only slightly as the concentration of the total metalloporphyrin was varied. Hence we may conclude that there is no split. Other evidence indicates that the molecules behave virtually as monomers. Hence we assumed the process  $\text{OP}_2 + e$



$\rightleftharpoons \text{RP}_2$  where the numbers of pyridine molecules are assigned on other evidence.

*Process XII*—Reductive titrations at high pH and in the presence of high constant concentration of total pyridine and constant pH gave slightly asymmetric titration curves. This is indicative of a split of the oxidant. For example, the data obtained at pH 11.22, when analyzed graphically, appeared to indicate the process  $\text{O}_2\text{P}_x + 2e \rightleftharpoons 2\text{RP}_x$ , or  $\text{P}_2\text{O}_2(\text{OH}^-)_2 + 2e$

TABLE III

*Titration of Pyridine Ferriprotoporphyrin with Reduced Phthiocol*

Phosphate buffer, pH 7.39; ionic strength, 0.13; total metalloporphyrin  $1 \times 10^{-4}\text{M}$ ; total pyridine 2.5 M; 30°. Analysis of data by Proposition XVI, giving  $n = 1$ ,  $d = 0.371$  ml.,  $y$  at 100 per cent reduction 4.565 ml.

Amount used	$y - d$	Reduction	$0.0601 \log \frac{S_r}{S_o}$	$E_h$	$E'_0$	Deviation from average
ml.		per cent				
0.50	0.13	3.10	-0.0898	0.1837	(0.0939)*	-0.0159
0.75	0.38	9.07	-0.0604	0.1664	(0.1060)	-0.0038
1.00	0.63	15.04	-0.0452	0.1542	0.1090	-0.0008
1.25	0.88	21.00	-0.0346	0.1444	0.1098	0.0000
1.50	1.13	26.97	-0.0260	0.1361	0.1101	0.0003
2.00	1.63	38.90	-0.0118	0.1216	0.1098	0.0000
2.50	2.13	50.84	0.0009	0.1086	0.1095	-0.0003
2.75	2.38	56.80	0.0071	0.1022	0.1093	-0.0005
3.00	2.63	62.77	0.0136	0.0958	0.1094	-0.0004
3.25	2.88	68.74	0.0206	0.0892	0.1098	0.0000
3.50	3.13	74.70	0.0282	0.0819	0.1101	0.0003
3.75	3.38	80.67	0.0374	0.0737	0.1110	0.0012
4.00	3.63	86.63	0.0488	0.0639	(0.1127)	0.0029
4.25	3.88	92.60	0.0660	0.0525	(0.1185)	0.0087
	4.194	100.00				
Average .....					0.1098	

\* Figures in parentheses omitted from the average.

$+ 2\text{H}^+ + 2\text{P} \rightarrow 2\text{RP}_2$  and when so treated there was obtained a fair conformity to the theoretical curve shown in Fig. 11. In that figure it is made obvious that the data will not conform to the curve for addition of 1 electron or that of 2 electrons without a split. If the assumption that the oxidant is split on reduction is correct, there should be a dilution effect such that  $\Delta E / \Delta \log S = -0.03$ . Fig. 11 (right) shows the slope to be  $-0.036$ . We conclude that the oxidant is dimeric and the reductant monomeric. Hence we specify the species formulated in Fig. 10, the number of pyridine molecules in each being assigned on other evidence. Inasmuch as the reductant

combines with pyridine more avidly than does the oxidant, there remains the possibility of complications which had best be examined with other systems in which it can be assured that both oxidant and reductant are saturated with the coordinating substance. Barron (4) found titration curves for  $n = 2$  but did not test for the dilution effect. Also Davies (7) found  $n = 2$  in his titration of nicotine iron mesoporphyrin in aqueous solution ( $n = 1$  in alcoholic water solution) and in his titration of pyridine iron hematoporphyrin but did not test for the dilution effect. The latter effect is more decisive than curve fitting.

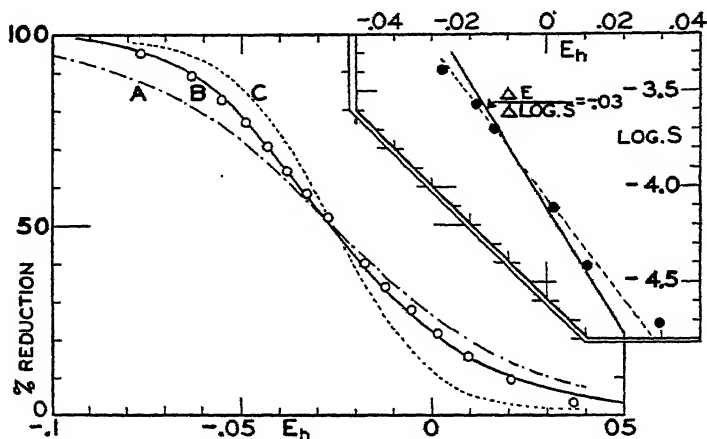


FIG. 11. Potentiometric test of Process XII. Titration of pyridine ferriprotoporphyrin by reduced phthiocol at pH 11.22; 30°. Total pyridine 2.5 M; total ferriprotoporphyrin  $1 \times 10^{-4}$  M. Curve A, theoretical for oxidant +  $e \rightleftharpoons$  reductant; Curve B, theoretical for (oxidant)<sub>2</sub> +  $2e \rightleftharpoons$  2 reductant; Curve C, theoretical for oxidant +  $2e \rightleftharpoons$  reductant. Inset, test of postulate (B) by dilution effect.

*Transition from Process I to Process XII*—If the Processes I and XII are as specified, there should be a gradual increase in the steepness of the reductive titration curves in the region of pH where the upper curve of Fig. 5 inflects. These effects were found in Barron's data and our own. Were the system one that lends itself well to very precise measurements, a detailed account of the progressive shift would have been attempted in spite of the complications arising from the participation of several species. Under the circumstances this was not attempted.

*Processes V and VIII Combined*—The potentials of the mid-points of reductive titration curves obtained with high, constant concentrations of pyridine and at different pH values are plotted in Fig. 5. At the higher pH values  $\Delta E/\Delta \text{pH} = -0.06$ , indicating clearly that for each electron concerned in reduction an oxidant has one more hydroxyl, or 1 less proton, per

iron atom. The fact that the potentials are much more positive than corresponding potentials in the absence of pyridine indicates that pyridine combines better with ferroprotoporphyrin than with ferriprotoporphyrin (see Proposition III (5)). At lower pH the slope is zero, indicating that there is then no change with respect to proton or hydroxyl. The point of inflection is at pH 9.13. This is *prima facie* evidence of an acid ionization exponent of an oxidant (see Propositions X and XI). The value differs widely from the estimated value 7.4 to 7.6 for ferriprotoporphyrin itself.

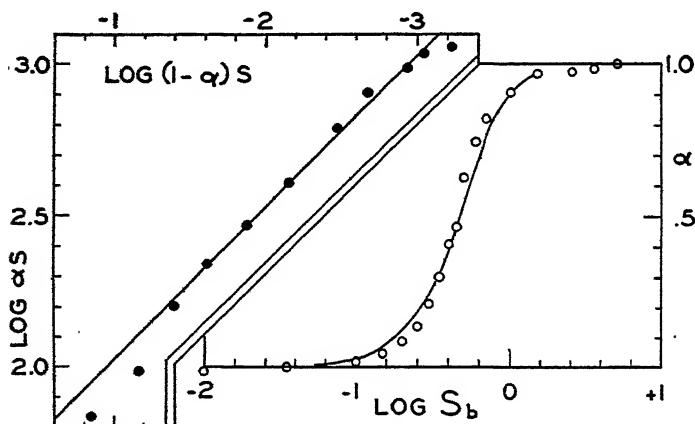


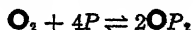
FIG. 12. Spectrophotometric test of Process II. Right-hand, total ferroprotoporphyrin  $9.18 \times 10^{-5}$  M.  $S_b$  = total pyridine. pH = 6.37;  $t = 30^\circ$ ; 560 m $\mu$ . Theoretical curve for  $[\text{O}_2][P]^4/[\text{OP}_2]^2 = 1.9 \times 10^{-8}$ . Half transformation at  $\log S_b = -0.32$ . Left-hand, dilution experiment. Ferroprotoporphyrin  $7.39 \times 10^{-5}$  to  $2.77 \times 10^{-4}$  M. pH 6.7. Pyridine 0.5 M.  $\alpha = [\text{OP}_2]/S$ ;  $S = [\text{OP}_2] + 2[\text{O}_2]$ . Line for  $\frac{\Delta \log (1 - \alpha) S}{\Delta \log \alpha S} = 2$ .

Presumably it pertains to the retained  $\text{OH}^-$ , the value of  $\text{pK}'$  shifting to higher values with increase of pyridine and reaching a limiting value when pyridine saturates the metalloprophyrin.

The marked contrast, shown in Fig. 5, between the cyanide and the pyridine iron protoporphyrin indicates clearly that pyridine competes poorly with hydroxyl for position at the "coordination center."

*Process II*—A spectrophotometric study of the addition of pyridine to ferroprotoporphyrin at pH 6.37 gave an association curve which was unsatisfactory for purposes of precise, objective analysis. On the other hand the slope of the curve, shown in Fig. 12, is steeper than the curve for the addition of two pyridines without a split and it shows such asymmetry as to suggest the splitting of a dimer on addition of pyridine. Accordingly

the critical dilution test was used. The results are shown in Fig. 12. The slope of the line gives  $c/a = 2$  for the process  $a\text{O}_m + bP \rightleftharpoons c\text{O}_nP_2$  (see comment on Proposition XII). We have concluded that a gross representation of Process II, with neglect of stepwise addition, is



with half transformation at  $\log [\text{total pyridine}] = -0.32$ .

*Process VIII*—The potentiometric data suggest almost conclusively that there is no essential change of pyridine ferroprotoporphyrin with change of pH in the region examined. This is confirmed by the fact that no change of absorption spectrum was observed in the visible region; see also Fig. 6 of Clark and Perkins' (9) data on pyridine ferrocoproporphyrin.

*Process XI*—On addition of pyridine to ferroprotoporphyrin a spectral change occurred and near  $560 \text{ m}\mu$  there was found a distinct peak. The association curve calculated with the data gave a distinct plateau, as if there were 100 per cent conversion. On further addition of pyridine the peak near  $560 \text{ m}\mu$  progressively shifted to lower wave-lengths. This suggests two distinct steps, as in the case of the addition of  $\text{CN}^-$ , but without that overlap of steps indicated for the cyanide case. Attempts to estimate the association curve for both steps were vitiated by drifts of optical density on the rise of the association curve. The half transformation point for the first step was estimated to be at  $\log [\text{pyridine}] = -2.5$  and of the second step  $-0.35$ .

In a rather bold analysis of similar data for the case of ferrocoproporphyrin Clark and Perkins (9) reached the conclusion that 2 molecules of pyridine coordinate with this metalloporphyrin. They indicated, however, some disconcerting facts and were unable to repeat the experiment because of exhaustion of available coproporphyrin. Our judgment of the significance of the present data is that 2 pyridine molecules coordinate *per iron atom*, accompanied by a split of the dimeric metalloporphyrin. The addition may well be stepwise. In any event the formulation in Fig. 10, which is suggested by the data, but not proved thereby, is consistent with other evidence of the natures of the reduced metalloporphyrin and its complex with pyridine.

*Process IX*—The nature of the compound formed when pyridine is added to a ferriporphyrin will be made the subject of subsequent comment. Certain anomalies will be noted; so that it behooves us to treat the experimental data with special care for the limitations of the method and a special effort to make the analysis of the data as objective as possible.

The optical densities observed at successive values of  $\log [\text{pyridine}]$  are shown by the centers of solid dots in Fig. 13. It is obvious that the optical density,  $D_2$ , at complete saturation of the metalloporphyrin with pyridine

is slightly uncertain but that it can be estimated fairly well by extrapolation of a curve of almost any one of the classical forms.  $D_0$ , the optical density of the metalloporphyrin solution, is as definite as the optical instrument (see (9)) permits. By the application of Proposition XII we conclude that the best working hypothesis is that 2 molecules of pyridine add to what we previously have concluded is a dimeric unit of the metalloporphyrin. How-

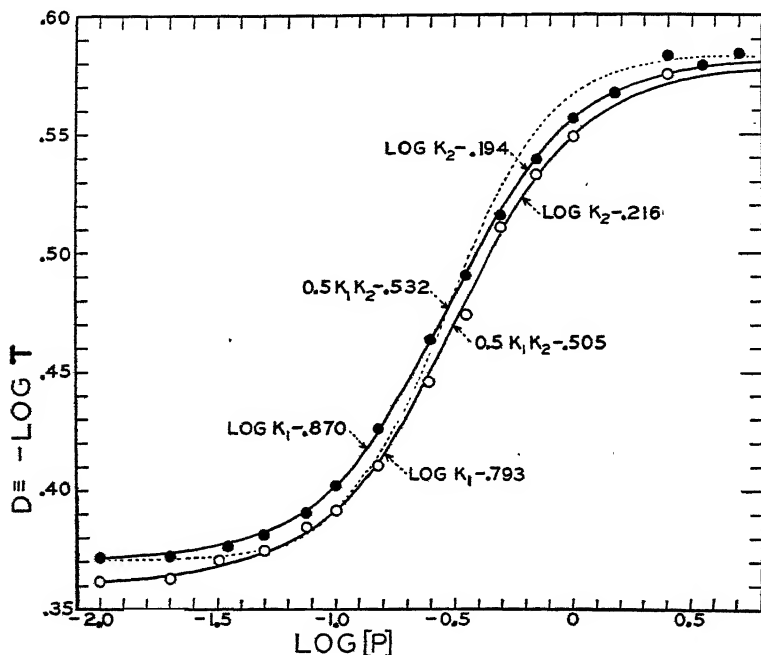


Fig. 13. Spectrophotometric test of Process IX. Total metalloporphyrin  $9.2 \times 10^{-4}$  M. Ordinate, observed density; abscissa, log [pyridine]. O, data observed on addition of pyridine at pH 11.2;  $m_{\mu} = 575$ . ●, data observed on addition of pyridine at pH 12.63;  $m_{\mu} = 575$ . Analysis of data by use of two experimental points and equation (6) with the assumption of  $D_1$  (see the text). Full lines, calculated for stepwise addition of 2 pyridine molecules per unit of metalloporphyrin and with  $K_2 > K_1$  (Case 4, p. 146); dotted line, theoretical for addition of 2 pyridines as by Case 1.

ever, the experimental curve is slightly more spread out than the curve for Case 2 (p. 146). By applying equation (6) to two points on the experimental curve on the left of Fig. 13 we calculate  $D_0 = 0.370$ ,  $D_2 = 0.583$  and assuming  $D_1 = 0.477$ , we find  $K_1 = 0.135$ ,  $K_2 = 0.64$ . With these constants the full curve, which runs in fair conformity with the experimental data (solid dots), was plotted (1 cm. cells were used).

It may be estimated that, if the constants are valid, the *maximal* concentration of the monopyridine complex is only 15 per cent of the total. It is to argue in an insignificant circle to say that this justified the arbitrary assignment of a reasonable extinction coefficient,  $\epsilon_1 = D_1/S$ , for doubling the value of  $\epsilon_1$  alters the estimated *maximal* concentration of the monopyridine complex by only about 1 per cent.

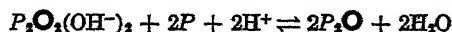
A comparable treatment of the data for the association of pyridine with ferriprotoporphyrin at pH 11.22 gave the curve running close to the circles (experimental) of Fig. 13.

It will be noted that a very high molar concentration of pyridine is required to approach saturation of the metalloporphyrin. It would require a very extensive investigation to evaluate all the corrections necessary for an application of a strict equilibrium equation. Inasmuch as these corrections are unknown, we have no confidence that the numerical values of the calculated constants are meaningful. They have, however, served to define the mid-points of the transformation. Also it is clear that 2 pyridine molecules add without a split of the dimer.

It should be noted that, if  $pK' = 9.1$  to  $9.2$  is the half transformation point in the conversion of the acid form of the dipyrindine complex to the alkaline form, pH 11.2 should be sufficiently high to convert about 99 per cent of the acid form to the alkaline form and then, if no further change were to take place, the mid-points of the curves of Fig. 13 should agree exactly. They do not. Unpublished data of other comparable systems suggest that the weak coordination of pyridine with a metalloporphyrin is somewhat sensitive to the state of aggregation of the metalloporphyrin. This, as influenced by specific salts, may account in part for such discrepancies as are evident in the displacement of one curve from the other in Fig. 13; but see the comment on p. 159 regarding a second ionization constant. Perhaps the curve at pH 12.6 is displaced from the other because of the involvement of a 2nd ion of ferriprotoporphyrin to a significant extent.

*Process V*—Repeated attempts to define precisely the nature of this transition by means of spectrophotometric data have given association curves approaching the form for  $pH = pK_a + \log (1 - \alpha)/\alpha$ , where  $\alpha$  is the ratio of the concentration of unionized form to that of total metalloporphyrin. Fig. 14 illustrates a case in which this simple relation describes the data fairly well, as it has similar data for other metalloporphyrins (see (7)).

On the other hand, consistency with the relations already decided tentatively requires consideration of the process



which involves a split that can be tested by a dilution effect. Let  $S = 2[P_2O_2(OH^-)_2] + [P_2O]$  and let  $\alpha = [P_2O]/S$ . Then for  $[P_2O_2(OH^-)_2]/$

$[P_2O] = K/[H^+]^2[P]^2 = K'$  (at constant pyridine concentration and constant pH) we have

$$\log S(1 - \alpha) - 2 \log \alpha S = C$$

or

$$\frac{d \log \alpha S}{d \log S(1 - \alpha)} = \frac{1}{2}$$

The lower curve of Fig. 14 shows that this prediction is fairly well confirmed.

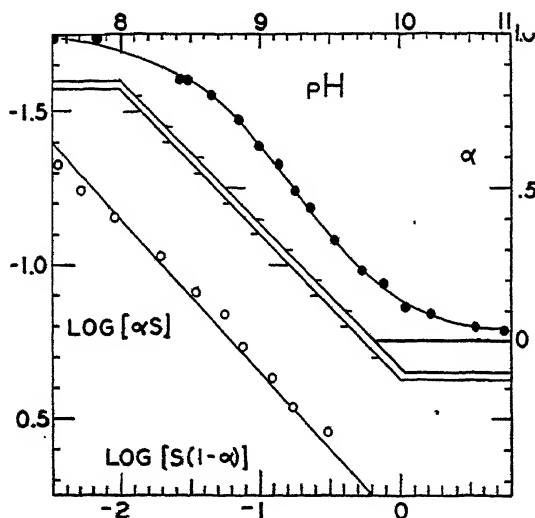


FIG. 14. Spectrophotometric tests of Process V. Right-hand curve,  $\alpha = [\text{acid form}]/S$ . Curve theoretical for  $\text{pH} = \text{pK}_a + \log (1 - \alpha)/\alpha$ . Total metalloporphyrin  $1.54 \times 10^{-4} M$  ( $\log S = -3.82$ ); total pyridine  $2.5 M$  ( $\log [P] = 0.40$ ); half transformation at pH 9.23. Left-hand curve, test of dilution effect

$$\frac{[P_2O_2(OH^-)_2][P]^2[H^+]^2}{[P_2OP]^2} = K$$

At constant pH and constant  $[P]$ ,

$$\frac{\Delta \log \alpha S}{\Delta \log S(1 - \alpha)} = \frac{1}{2}$$

We have here an interesting example of the limitations of particular experimental methods. The electrometric data summarized in Fig. 5 show clearly that there is a change of 1 proton per iron atom. Therefore, if we are to accept the evidence that the pyridine complex is dimeric and the pyridine does not replace  $OH^-$ , we should specify two  $OH^-$  groups in the

dimer. As noted, the spectral data appear to conform fairly well in this and other cases with a curve of the first order for addition of proton. Such conformity does not reveal a splitting of the dimer when protons and pyridine add in the less alkaline solutions. The split is revealed only by the dilution experiment and this does not show decisively the number of protons involved. Tests at concentrations of pyridine less than those necessary to approach saturation are rendered very uncertain of interpretation because of the involvement of several species.

It should be noted, however, that the analysis of the spectrophotometric data on the assumption of but two absorbing species is precarious. Indeed elaborate series of absorption curves of ferricoproporphyrin in solutions of various pH, and concentrations of pyridine short of a close approach to saturation, showed that no consistent pH curves could be obtained with data at *different* wave-lengths. This, irrespective of our prediction that there should be several absorbing species, is indicative of more than two species.

*Attempts to Check by Products of Constants*—The equilibrium constant for Process IX should equal the product of the constants for Processes 6, II, and V (see Fig. 10) if the equilibria relations are written as follows:

$$\frac{[(\text{OOH}^-)_2][P]^2}{[P_2\text{O}_2(\text{OH}^-)_2]} = \frac{[(\text{OOH}^-)_2][\text{H}^+]^2}{[\text{O}_2]} \times \frac{[\text{O}_2][P]^4}{[P_2\text{O}_2]^2} \times \frac{[P_2\text{O}_2]}{[P_2\text{O}_2(\text{OH}^-)_2][P]^2[\text{H}^+]^2}$$

(IX)
(6)
(II)
(V)

We find it impracticable to make this check for the following reasons. To obtain saturation of the metalloporphyrin with pyridine so that in the study of Process V there will remain insignificant proportions of other species it is necessary to have  $[P]$  very large. The numerical value of  $[P]$  used in the studies then is not critical as it should be to arrive at a value for the equilibrium constant of Process V. As already mentioned, an attempt to obtain consistent values for this constant in the case of ferricoproporphyrin (unpublished data) failed because there are present too many species, and calculations at different wave-lengths did not agree. Furthermore there are unexplained small differences in the evaluations of the constant for Process IX.

#### IV. Systems Containing Both Pyridine and Cyanide

Anson and Mirsky (32) described compounds in which both globin and cyanide were combined with ferroprotoporphyrin, and Hill (33) described compounds in which both nicotine and cyanide were combined with ferroprotoporphyrin. Hill also showed that the latter are formed at such concentrations of nicotine and cyanide that insignificant amounts of a complex are formed by nicotine, or cyanide, alone. Drabkin (34) has reported a simi-



lar phenomenon which occurs when ferriprotoporphyrin is subjected to the action of a mixture of cyanide and pyridine. We have observed that the pyridine-cyanide ferriprotoporphyrin complex is formed when the concentration of either coordinating substance is so low that, if either were present alone, no appreciable dicyanide or dipyridine ferriprotoporphyrin would be formed. Also, the higher the concentration of the one, the lower is the concentration of the other that is necessary to form the mixed compound; this is within limits, of course.

It is obvious that the case is not one of simple competition in the formation of those complexes that result from the addition of either  $\text{CN}^-$  or pyridine alone. Were this the case, the mixture of the pyridine metalloporphyrin and of the cyanide metalloporphyrin should be roughly predictable from the experimentally determined constants already determined. Such a predic-

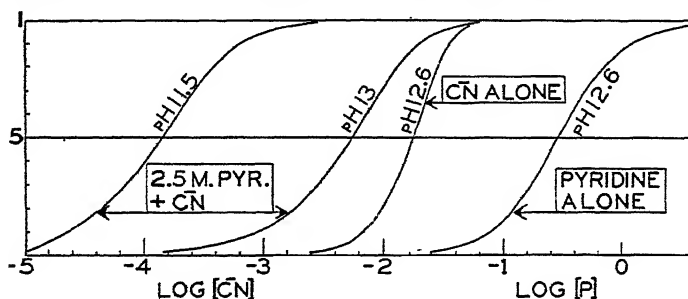
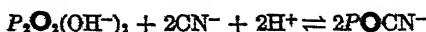


FIG. 15. Comparison of association curves for the formation of dipyridine ferriprotoporphyrin and dicyanide ferriprotoporphyrin at pH 12.6 and of the pyridine-cyanide ferriprotoporphyrin at pH 13 and 11.5.

tion would be in violent conflict with the fact that in the presence of pyridine which, if alone, coordinates very weakly with ferriprotoporphyrin, cyanide is bound more firmly than in the absence of pyridine and in the presence of  $\text{CN}^-$  pyridine is bound more firmly than in the absence of cyanide. This is revealed in perspective by Fig. 15. The only assumption that seems reasonable is that an entirely new species is formed. The data can be formulated well with the assumption that this new species is  $\text{P}^+\text{OCN}^-$ .

A preliminary study of the system was made by keeping the pyridine concentration at 2.5 M (which, if present alone, is sufficient to approach complete conversion of ferriprotoporphyrin to the dipyridine complex) and adding KCN. In Fig. 16 the sigmoid curve has the theoretical form for the equilibria of the following process at constant pH.



The upper left-hand curve summarizes a dilution experiment at constant pH. Except for three points at the lower concentrations the trend is clear. The

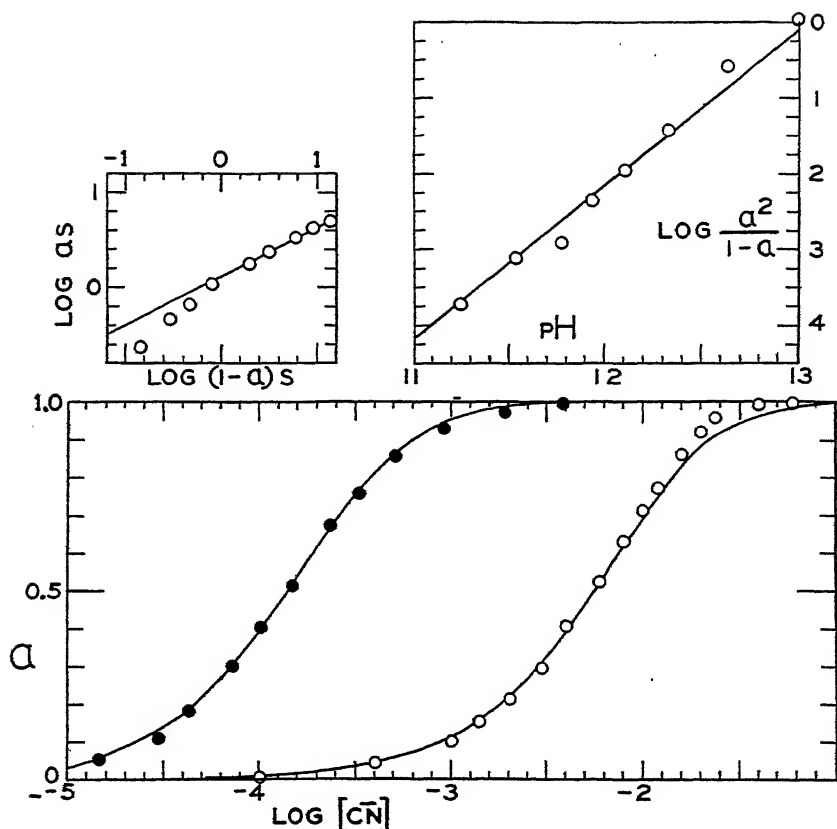


FIG. 16. Formation of monocyanide-monopyridine ferriprotoporphyrin from dipyrindine ferriprotoporphyrin. Lower sigmoid curves, lines theoretical for  $P_2O_2(OH^-)_2 + 2CN^- + xH^+ \rightleftharpoons 2P\bullet CN^-$ ; total ferriprotoporphyrin  $9.4 \times 10^{-5} M = S$ ; total pyridine 2.5 M. ●, data at pH 11.53; ○, data at pH 13.03. Cyanide concentration corrected for bound cyanide. Upper left-hand curve, test of dilution effect (see the text); pH 13.03;  $[P] = 2.5 M$ . Upper right-hand curve, test of pH effect; pyridine 2.5 M;  $S = 9.4 \times 10^{-5} M$ ; total cyanide  $9.97 \times 10^{-4} M$ .

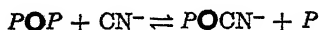
straight line is theoretical for the dilution effect at constant pH. There should be also a pH effect such that

$$\frac{\Delta \log \frac{\alpha^2}{1-\alpha}}{\Delta pH}$$

where  $\alpha$  is  $[P\bullet CN^-]/S$ . This effect is shown by the upper right-hand curve. The straight line is theoretical for the above relation.

The absorption spectrum of the compound postulated to be  $P\bullet\text{OCN}^-$  was found to be invariant from pH 7.64 to 13.0.

As pH decreases, there should be expected a transition to equilibrium for the process



The difficulty of making a direct test of the equilibrium is that the cyanide is bound so strongly as to make uncertain the distinction between total cyanide and free  $\text{CN}^-$ . This exceptionally strong binding of  $\text{CN}^-$  in the pyridine complex, in contrast to the strong but *relatively* weak binding in the dicyanide

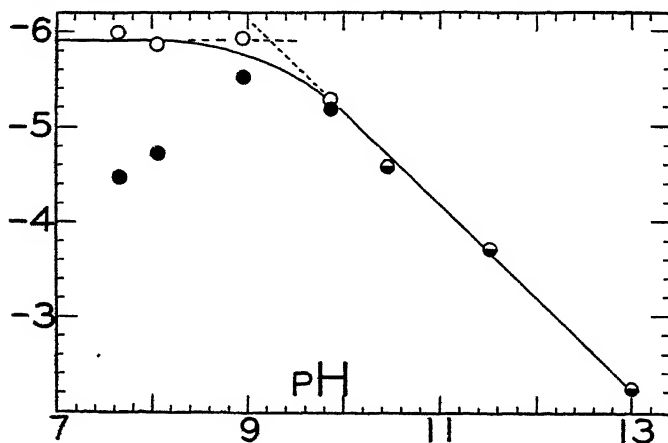


FIG. 17. Relation of  $\log [\text{CN}^-]$  and  $\log ([\text{CN}^-] + [\text{HCN}])$  to pH in the conversion of dipyrroline ferriprotoporphyrin to monopyrroline-monocyanide ferriprotoporphyrin. Abscissa, pH; ordinate,  $\log [\text{CN}^-]$  and  $\log ([\text{CN}^-] + [\text{HCN}])$ . ○, data for  $\log [\text{CN}^-]$ ; ●, data for  $\log ([\text{CN}^-] + [\text{HCN}])$ ; ●,  $[\text{CN}^-]$  approximates  $[\text{CN}^-] + [\text{HCN}]$ .  $S = 9.39 \times 10^{-4} \text{ M}$ ;  $[\text{pyridine}] = 2.5 \text{ M}$ .

complex, is indicated by Drabkin's (34) observation that at high hemin concentration and over a limited range of pH the spectral change is complete when 1 cyanide ion per iron atom has been added. We also had found this and studied the reaction over a wide range of pH. Low concentrations of metalloporphyrin were used to obtain conditions favoring partial binding of cyanide. From partial association curves there were estimated the concentrations of  $\text{CN}^-$  required for half transformation to the species  $P\bullet\text{OCN}^-$ . In Fig. 17 the logarithms of the free cyanide ion concentrations required for such half transformation are plotted against pH.

Presumably  $\alpha$ , as determined spectrophotometrically, is

$$\frac{[P\bullet\text{OCN}^-]}{2[P_2\text{O}_2(\text{OH})_2] + [POP] + [P\bullet\text{OCN}^-]}$$

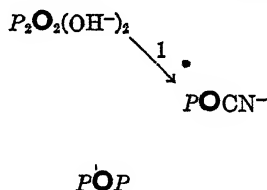
The intersection found is at pH 9.2, which, with due consideration of the difficulties involved, is in fairly satisfactory agreement with the value 9.34 estimated by Fig. 14 or 9.13 of Fig. 5.

The cases reported by Drabkin (34) are special ones at pH values at which competition by hydroxyl ions plays an insignificant rôle. Thus we estimate that at pH 8.9 only about 6 per cent of added  $\text{CN}^-$  remains uncombined, but as the pH is increased or decreased the uncombined fraction of  $\text{CN}^-$  increases.

Much more exhaustive studies should be made with a neutral coordinating substance that does not require the high concentrations required of pyridine to approach complete conversion to the base complex.

We tentatively conclude that  $\text{CN}^-$  and not  $\text{HCN}$  combines to form the pyridine-cyanide ferriporphyrin, that there is not the ordinary competition between pyridine, cyanide, and  $\text{OH}^-$  for position in the coordination shell to form the dipyridine and dicyanide complexes, but that  $\text{CN}^-$  replaces  $\text{OH}^-$  and 1 pyridine is left in the monomer  $P\text{OCN}^-$ .

*Check by Combination of Constants*—For the cycle



we shall find it convenient to formulate the equilibria as follows:

$$\begin{aligned}
 K_1 &= \frac{[P\text{OCN}^-]^2}{[P_2O_2(OH^-)_2][H^+]^2[CN^-]^2} \\
 K_2 &= \frac{[P\text{OP}][CN^-]}{[P\text{OCN}^-][P]} \\
 K_3 &= \frac{[P\text{OP}]^2}{[P_2O_2(OH^-)_2][H^+]^2[P]^2}
 \end{aligned}$$

For Process 1 at  $\log S = -4.03$  and pH 13.03,  $[P\text{OCN}^-]/[P_2O_2(OH^-)_2] = 1$  when  $\log [CN^-] = -2.26$ . Hence  $\log K_1 = -4.03 - 2(-2.26) + 2(13.03) = 26.55$ .

For Process 2 at  $\log [P] = 0.40$ ,  $[P\text{OP}]/[P\text{OCN}^-] = 1$  when  $[CN^-] = -5.92$ . Hence  $\log K_2 = -5.92 - 0.40 = -6.34$ .

For Process 3 at  $\log S = -3.82$  and  $\log [P] = 0.40$ ,  $[P\text{OP}]/[P_2O_2(OH^-)_2] = 1$  when pH = 9.23. Hence  $\log K_3 = -3.82 - 2(0.40) + 2(9.23) = 13.84$ .

$K_1/K_3 = (1/K_2)^2$  or  $\log K_1 - \log K_3 = -2 \log K_2$ ; that is,  $26.55 - 13.84$  should equal  $-2(-6.34)$ . The first value is 12.71 and the second 12.68,

a check which is well within the experimental errors and better than could be expected.

#### A CORRELATION WITH SPECTRAL DATA

Having been led by analyses of association curves and other data to ascribe monomeric units to certain of the compounds and dimeric units to others, we find interesting the correspondence between these deductions and the grouping of spectrophotometric absorption curves, as exhibited in Fig. 18.

The data shown in Fig. 18 were determined at 30° with the spectrophotometer described by Clark and Perkins (9). The concentrations of the metalloporphyrin and its complexes were in the range 0.04988 to 0.05029 mM, except for the dipyrindine ferroprotoporphyrin which was 0.02012 mM. From the concentration and length of cuvettes (certified by the American Instrument Company to be 10.017 mm.) the absorption coefficients for 1 mM concentration (neglecting any dilution effect) and 1 cm. were calculated and plotted in Fig. 18. The spectral interval isolated was of the order of 4  $m\mu$  at 546.1  $m\mu$  (Hg line); so that the sharper peaks are not maximal.

Obviously the curves fall into two groups, one of which pertains to those substances which presumably have dimeric units (at the left), the other group pertaining to those which presumably have monomeric units (at the right). Of particular interest are the pyridine ferriprotoporphyrin complexes in alkaline and approximately neutral solutions. The complex in neutral solution has been assigned a monomeric unit and has a sharper and higher peak than the complex in alkaline solution, which presumably has a dimeric unit.

To avoid confusion the curve for the monocyanide-monopyridine ferriprotoporphyrin has not been included in Fig. 18. This compound has been assigned a monomeric unit and in conformity with the relation discussed the optical density at maximum is nearly twice that of the dipyrindine ferriprotoporphyrin and is of the order of that of the monomeric dicyanide ferriprotoporphyrin (compare the curves in Figs. 1 and 2 of Drabkin's paper (34)).

#### COMMENTS ON SOME PROBLEMS OF STRUCTURE

We have no direct evidence of the structure of any of the species. We have been led to postulate compositions by following objective methods of analyzing data. The conclusions pose certain problems of bonding to which attention is drawn.

The evidence as a whole indicates that several of the species exist in solution as micellae within which there are dimeric units. We need not repeat here the cases in which one type of experiment reveals dimerization or the splitting of a dimer and another type of experiment does not. We think it of general interest, however, to note that we have in these cases a revelation of the limitations of experimental methods with respect to the detail that they can disclose.

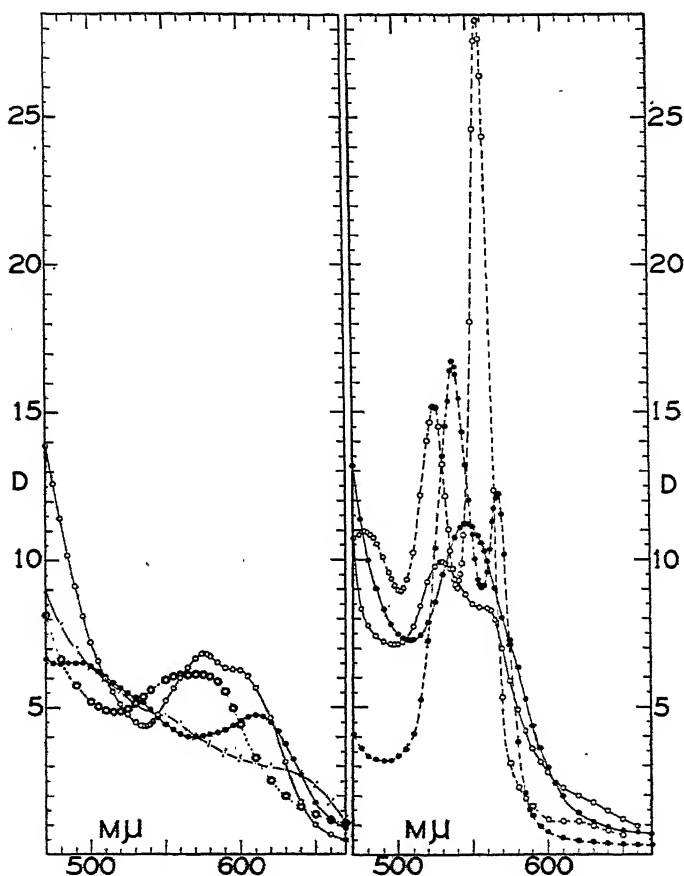


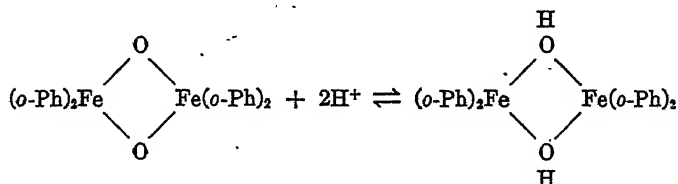
FIG. 18. Spectrophotometric curves of the compounds which presumably are dimeric (left) and of those which presumably are monomeric (right). Ordinate, optical density calculated for 1 mm solution of metalloporphyrin in a 1 cm. cuvette; see the text for the conditions of measurement. Left-hand diagram (of "dimers"): ●, ferriprotoporphyrin in 0.02 M KOH; ——— ferriprotoporphyrin in phosphate buffer of pH 6.42; ○, ferroprotoporphyrin in 0.02 M KOH; ○, ferriprotoporphyrin in 0.02 M KOH + 4.97 M pyridine. Right-hand diagram (of "monomers"): ●, solid line, ferriprotoporphyrin in 0.02 M KOH + 0.2 M KCN; ●, dash line, ferriprotoporphyrin in 0.02 M KOH + 0.2 M KCN; ○, solid line, ferriprotoporphyrin in phosphate buffer, pH 7.7, + 4.97 M pyridine; ○, dash line, ferroprotoporphyrin in 0.2 M KOH + 4.97 M pyridine or phosphate buffer, pH 7.7, + 4.97 M pyridine.

Starting with the simplest concept, we may consider the iron of ferriprotoporphyrin in moderately alkaline solution to have its six coordinating bonds engaged by the 4 pyrrol nitrogens, a water molecule, and a hydroxyl ion.

Provision must now be made for the bonding of 2 such molecules in a dimeric unit.

The data indicate that on addition of ligand the 2 iron atoms act independently of one another; so that it is improbable that the bond is directly between the irons.

Gaines, Hammett, and Walden (35) obtained evidence in the case of the *o*-phenanthroline complex of ferric iron for bonds represented as follows:



This system behaved as an acid system, with stepwise addition or loss of protons. Now there are some signs that ferriprotoporphyrin loses a 2nd proton in a region of pH much higher than that of the solutions we have examined. If so, an analogy might be drawn. But difficulties are encountered immediately. We would be led by this postulate to assume that addition of cyanide would require (in moderately alkaline solution) replacement

of the  $\text{—O—}$  bond and the  $\text{—O—}$  bond. Presumably the difference in bond energies would be reflected in stepwise addition of  $\text{CN}^-$ . No evidence of *distinct* steps appears in our data. It might be obscured by the over-all complexity of the change.

Weisiger (36) suggests one bond of the type indicated by  $\text{H}_2\text{O}\ddot{\text{O}}\ddot{\text{O}}\text{H}_2\text{O}$ . This would account nicely for the addition of 2 pyridine molecules by replacement of the 2 water molecules and preservation of a dimer. It would obviate the necessity of postulating seven coordination bonds as suggested by Clark and Perkins (9). This suggestion has been quoted by others (37, 38) without due emphasis upon the following fact. Clark and Perkins were formulating their own experimental data as objectively as was feasible and in the simplest terms that seemed consistent with the data. Thereby they were led to an apparent anomaly that required resolution. The suggestion of seven coordination bonds was a tentative resolution admitted to be in conflict with accepted views. Weisiger's suggestion leaves  $\ddot{\text{O}}$  as the potential binder of 2 protons and, inasmuch as these would add in close juxtaposition, it may be assumed that the steps would be widely separated unless the addition of 1 proton automatically creates a unique situation. According to Davies' finding (see p. 159) there are, to be sure, widely separated steps of proton exchange but our data call for the addition of 1 proton per iron atom

*within the range of pH intermediate between the two ionization exponents and within this range there is no evidence of stepwise addition of protons.*

Our data have led us to the conclusion that pyridine ferriprotoporphyrin contains 1 pyridine molecule and 1 hydroxyl ion per iron atom, but in a dimer. If the coordination number 6 for iron is to be preserved in the formulation of the structure of this dimer, the bonding (other than by relatively weak van der Waals forces) presumably involves  $\text{OH}^-$  groups or their equivalent. Only when the latter are eliminated, presumably by conversion to  $\text{H}_2\text{O}$ , can more than 1 pyridine per iron atom add. Then it does so with the splitting of the dimer. Hence the bonds are not between pyridine molecules.

If  $\text{OH}^-$ , or its equivalent, exerts a bonding effect in dimers present in alkaline solutions, to what may the bonding be attributed in dimers present in the neutral solutions? Presumably the bonds are between water molecules. When these are replaced by  $\text{CN}^-$ , or by pyridine, the dimer is split.

Superficially the case of the pyridine-cyanide complex may be approached first on the basis of the *formal electric charges at the coordination center*. The formal charge with  $\text{Fe}^{+++}$  and  $2\text{N}^-$  alone is  $1+$ . This is not changed by addition of 2 neutral pyridine molecules. The formal charge is changed to  $1-$  on addition of  $2\text{CN}^-$ . When 1 pyridine and  $1\text{CN}^-$  are added, the formal charge is zero and quite different from the other cases. According to Drabkin (34) reduction of the pyridine-cyanide ferriprotoporphyrin results in a spectrum nearly the same as that of the dipyridine ferroprotoporphyrin. This would be expected, because the unique situation obtaining in the case of the monocyanide-monopyridine ferriporphyrin no longer holds for the complex with ferroprophyrin and because the cyanide concentration which Drabkin used was too low to compete with the large concentration of pyridine for position in the *ferrous* compound.

The planar configuration and general symmetry of metalloporphyrins are not unlike comparable features of phthalocyanines. Sulfonic acid derivatives of the latter are included in Sheppard and Geddes' (39) treatment of the optical evidence of aggregation and dimerization of dyes in aqueous solution. In addition to the forces of labile electrons of such resonating molecules, which forces could hold the molecules in aggregates, a water molecule is suggested as possibly accounting for the dimerization of dyes in aqueous solutions.

We may imagine the molecules of a metalloporphyrin to be stacked like pancakes in variable, large aggregates with intervening water molecules, or oxide bridges. A formulation of equilibrium based on this conception would have to be a statistical treatment of random attack by protons and coordinating substances. A statistical treatment would have to account for phenomena which we have interpreted as dimerization and the splitting of dimers.



At the moment the possibilities appear too numerous to select with a view to bringing a statistical formulation within the reach of experimental test by the limited methods used in the work here reported.

Some of these comments are gratuitous and would be omitted were it not that they serve to focus attention upon anomalies that emerge from the tentative conclusions to which objective analyses of the data have led. They also focus attention upon limitations of particular experimental methods; a matter of general interest.

We thank Miss Marie Perkins for assistance in the preparation and careful standardization of solutions.

#### SUMMARY

There are presented evidences that the molecules of ferriprotoporphyrin (heme) in aqueous solution are in large, variable micellae.

By means of potentiometric titrations and spectrophotometry there were studied equilibria in exchange of protons, electrons, and coordinating substances, particularly cyanide ions and pyridine. Cycles of changes were investigated in order to reach consistent conclusions.

It appears that energetic interaction between parts of the micellae is so weak that some data do not reflect it and certain relations may be formulated as if the molecules were dispersed. Other data require the assumption of dimeric units, such dimers being split in the cases cited below.

Direct and indirect evidence indicates that the carboxyl groups of the porphyrin are completely ionized in the pH range considered. The following references to  $\text{OH}^-$  and addition of protons thereto concern the coordination shell of iron.

Analyses of data have led to the following specific conclusions regarding the compositions of species in terms of which the equilibria have been formulated for specification of the equilibrium constants numerically established.

In moderately alkaline solution ferriprotoporphyrin has a dimeric unit containing 1 hydroxyl ion per iron atom. Presumably because of little interaction between the 2 iron atoms the compound behaves on titration as if it were a univalent base,  $\text{pK}_a$  being between 7.4 and 7.6. The dimeric unit is retained in neutral solution.

Although the dimeric unit appears to be retained in ferroprotoporphyrin, reduction to this species is as if 1 electron per unit were required, again presumably because of little interaction. The reductant contains no hydroxyl ion, as is made evident by the fact that the electrode potential of a fixed mixture of oxidant and reductant varies with pH according to  $\Delta E_s/\Delta \text{pH} = -0.0601$  at  $30^\circ$  (confirmed spectrophotometrically by invariance of spectrum with change of pH).

HCN does not combine with either the oxidized or reduced iron porphyrin. Cyanide ion combines with either. In combining with the oxidant, it replaces  $\text{OH}^-$ . This is accompanied by a split of the dimer and the formation of monomeric dicyanide ferriprotoporphyrin. Cyanide ion also causes a split of dimeric reductant and formation stepwise of monomeric dicyanide ferroprotoporphyrin. When a fixed mixture of oxidant and reductant is saturated with  $\text{CN}^-$ , the electrode potential does not vary with pH. A result of the competition between  $\text{OH}^-$  and  $\text{CN}^-$  for place in the coordination shell of the oxidant is that while the hydroxyl ion concentration is high  $\text{CN}^-$  combines more firmly with the reductant than with the oxidant but the reverse is true when  $\text{CN}^-$  is relieved of this competition at relatively low pH.

In *neutral* solution the addition of pyridine results in the splitting of the dimeric unit of ferriprotoporphyrin with the formation of monomeric dipyridine ferriprotoporphyrin. On reduction this takes up 1 electron per iron atom to form monomeric dipyridine ferroprotoporphyrin.

In alkaline solution the addition of pyridine results in a dimeric dipyridine complex retaining, presumably, 1 hydroxyl ion per iron atom. This behaves on titration as if it were a univalent base with  $\text{pK}_a$  at about 9.1 to 9.2 when the system is practically saturated with pyridine. This is split on addition of protons to form the compound mentioned in the foregoing paragraph. On reduction at high concentration of pyridine it appears that 2 electrons, 2 protons, and 2 pyridine molecules add to the dimer, split it, and form monomeric dipyridine ferroprotoporphyrin. As a result of the absence of  $\text{OH}^-$  in this reductant a fixed mixture of reductant and oxidant in the presence of high concentration of pyridine shows  $\Delta E_h/\Delta \text{pH} = -0.0601$  at  $30^\circ$  while the pH is significantly greater than 9.2 but the potential becomes invariant with pH at lower values of pH at which the oxidant no longer retains  $\text{OH}^-$ .

Although the equilibrium constants are functions of pH, it may be said in general that the concentration of pyridine at which ferriprotoporphyrin is half converted to the pyridine complex is very much higher than the concentration of cyanide ion required for half transformation to the dicyanide complex. Specific data are reported.

When both pyridine and cyanide ions are present, there is formed a coordination complex at cyanide ion concentrations much less than would be required to form the dicyanide complex in the absence of pyridine. The data are in violent conflict with the assumption that there is formed a mixture of those dicyanide and dipyridine complexes which are formed when either cyanide or pyridine, respectively, is present alone. The data are accounted for quantitatively on the assumption that an entirely new species is formed, namely monomeric monocyanide-monopyridine ferriprotoporphyrin. The function of pH in the formation of this compound has been described.

Several of the conclusions mentioned above have been reported previously but now are brought into a fairly consistent system.

There have emerged certain problems of structure which are discussed. Differences between samples of hemin chloride are noted.

Considering as a whole any one of the systems containing a metalloporphyrin and a coordinating substance, we may find circumstances in which there are integrated the free energies of proton exchange, of oxidation-reduction, and differences between the free energies of association of the coordinating substance with the metalloporphyrin in different states. To some extent there is also integrated the free energy pertaining to dimerization. The free energy of aggregation to micellae is reflected faintly or not at all in the data. If faintly, it may account in part for the slight irregularities among the data.

It is emphasized that the above conclusions have been reached by following objective analyses of data so far as feasible and with the simplest assumptions that lead to consistency.

In so far as such an artificial system is comparable to natural systems, the outline given serves to show something of the complexities of natural systems and also a step toward resolution.

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# THE MECHANISM OF IODINE CONCENTRATION BY THE THYROID GLAND: ITS NON-ORGANIC IODINE-BINDING CAPACITY IN THE NORMAL AND PROPYLTHIOURACIL-TREATED RAT\*

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In normal rats maintained on an adequate iodine intake, the iodine concentration in the thyroid gland is about 10,000 times as great as that in plasma, a finding that illustrates the remarkable iodine-concentrating capacity of this tissue (1). The fact that almost all of the iodine in the normal gland is organically bound (2, 3) would suggest that this concentrating capacity is dependent upon the gland's ability to convert inorganic iodine to diiodotyrosine and thyroxine. Although this view is supported by the rapid rate at which an injected *tracer* dose of inorganic radioactive iodide is organically bound by the gland (3), it does not account for the iodide concentration by thyroid tissue when large amounts of iodide are introduced into the animal. Thus, Leblond (4) observed that in guinea pigs that received 500  $\gamma$  of labeled iodide per 100 gm. of body weight the largest part of the iodine fixed by the gland remained in the form of inorganic iodide for some time. Also, Lein (5) reported that most of the radioiodine taken up by the thyroids of rabbits during the first 10 minutes after the intravenous injection of 35  $\gamma$  of labeled iodide was still in the form of iodide.

The first demonstration that the mechanism of iodine concentration by thyroid tissue can be completely separated from synthesis of diiodotyrosine and thyroxine was made in this laboratory with the aid of surviving slices of thyroid tissue. Franklin *et al.* observed that in the presence of  $10^{-3}$  M thiouracil surviving thyroid slices failed to convert inorganic iodide to thyroxine and diiodotyrosine but retained their capacity for concentrating iodine (6). *p*-Aminobenzoic acid, thiourea, and sulfanilamide were shown by Franklin *et al.* (6) and by Schachner *et al.* (7) to behave in a similar manner.

Subsequently, in intact animals, McGinty (8) and Astwood (9) found that thiouracil treatment does not abolish the iodine-concentrating capacity of the gland, even though it completely blocks the production of the thyroid hormone. The iodine taken up under these conditions, as shown by

\* Aided by grants from the United States Public Health Service and the Committee for Research in Endocrinology of the National Research Council.

Vanderlaan and Bissel (10), does not remain in the gland for long, the maximum concentration being reached within the first half hour after the iodine injection.

The present communication deals with the non-organic iodine-concentrating mechanism of the thyroid gland. The use of both radioactive and chemical measurements made possible a more complete investigation of this subject than had heretofore been reported.

#### EXPERIMENTAL

Male rats that weighed 200 to 300 gm. were used throughout. They were maintained on a stock diet that contained 0.3  $\gamma$  of iodine per gm. Propylthiouracil was administered by mixing it with the diet to the extent of 0.10 or 0.15 per cent. Both the control and the goitrogen-containing diets were fed *ad libitum* up to the time that the animals were sacrificed. Radioactive iodine was administered by the intraperitoneal route.

*Fractionation of Thyroid Iodine*—The thyroids from each rat were homogenized with 1 cc. of cold 10 per cent trichloroacetic acid in a small glass homogenizer. The homogenate was centrifuged, the precipitate washed once with 5 cc. of 5 per cent trichloroacetic acid, and the mixture again centrifuged. The supernatants from each rat sample were combined to form the inorganic iodine fraction. The precipitate was dissolved in 1 cc. of 2 N NaOH for determination of protein-bound (presumably organic) iodine. Radioactivity was determined by a scale-of-eight Geiger-Müller counter equipped with a thin mica window tube. Iodine determinations were carried out as previously described (11).

#### *Effect of Propylthiouracil Feeding on Iodine Content of Thyroid*

The thyroids of rats fed a diet containing 0.15 per cent propylthiouracil for 16 days were 3 or 4 times the normal size (Table I). These enlarged glands contained only about 1 to 2 mg. per cent of total iodine compared with the normal concentration of 35 to 60 mg. per cent. The iodine lost through the action of the goitrogen was entirely organic.

#### *Uptake of Radioiodide by Thyroids Made Goitrous by Prolonged Administration of Propylthiouracil*

Rats were fed a diet containing 0.10 or 0.15 per cent propylthiouracil for 15 to 17 days. At the end of this period the uptake by their thyroids of an intraperitoneally injected dose of  $I^{131}$  was compared with that of control rats that were fed the same diet to which no propylthiouracil had been added. Two types of experiments were conducted. In the first, a sample of radioiodide containing no carrier was injected. This sample is referred

TABLE I  
*Effect of Propylthiouracil on Iodine Content of Thyroid Gland of Rat*

Treatment	Weight of rat	Weight of thyroids	Thyroid iodine			
			Organic iodine fraction		Inorganic iodine fraction	
			Iodine	Iodine concentration	Iodine	Iodine concentration
	gm.	mg.	$\gamma$	mg. per cent	$\gamma$	mg. per cent
Controls	236	16	6.6	41	0.22	1.4
	235	18.5	11.1	60	0.21	1.1
	224	18	7.4	41	0.20	1.1
	320	25	8.8	35	0.29	1.2
Diet containing 0.15% propylthiouracil for 16 days	230	51	0.50	0.98	0.14	0.27
	210	60	0.26	0.43	0.16	0.27
	160	51	0.45	0.88	0.24	0.47
	239	79	0.21	0.27	0.42	0.53
	279	57	0.22	0.39	0.66	1.2

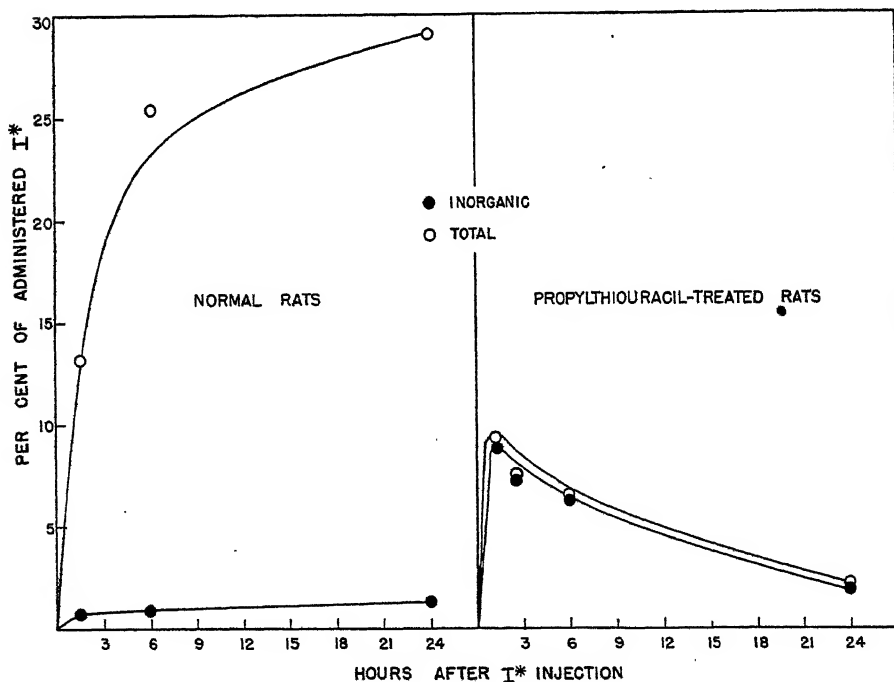


FIG. 1. The uptake of a tracer dose of radioactive iodide by the thyroids of normal and propylthiouracil-treated rats. The latter received a diet containing 0.10 per cent propylthiouracil for 17 days.



to here as a tracer dose. In the second, a sample of radioiodide containing 100  $\gamma$  of iodide carrier was injected.

*Experiments with Tracer Dose*—The inorganic radioiodide that entered the *normal* gland (Fig. 1) was rapidly converted to organic iodine, and throughout the entire period of observation the latter constituted more than 95 per cent of the total  $I^{131}$  of the gland. The amount of organically bound  $I^{131}$  contained in the gland continued to increase with time, and the highest value, namely 30 per cent of the injected radioactive iodine, was found at the 24 hour interval.

In the *goitrous* gland, on the other hand, practically all of the radioactive iodide that entered the gland remained as inorganic. The maximum uptake of  $I^{131}$ , namely 9 per cent, was found 1 hour after the injection; thereafter the  $I^{131}$  content of the gland decreased continuously.

*Experiments with 100  $\gamma$  of Labeled Iodide*—In this experiment the uptake of the radioactive iodide by the *normal* gland was slower than when the tracer dose was injected. Only 0.5 per cent was taken up by the 1 hour interval and 2.5 per cent by 26 hours. At the 1 hour interval, approximately 50 per cent of the thyroid's radioiodine was recovered in the inorganic fraction. This value should be compared with that observed in normal rats after injection of a tracer dose of radioiodide, in which case it was found that very little of the gland's radioiodine remained in the form of inorganic iodine, even at a very early interval after the injection.

The curve depicting the uptake of  $I^{131}$  by the *goitrous* gland when 100  $\gamma$  of labeled iodide were injected (Fig. 2) resembles quite closely that observed with the tracer dose (Fig. 1). Maximum uptake by *goitrous* glands amounted to somewhat more than 10 per cent of the 100  $\gamma$ , and this occurred within 30 minutes. Thereafter the percentage of the injected iodide present in the gland decreased steadily, and in 26 hours only 0.5 per cent was found there. Practically all of the radioiodine at all intervals was found in the inorganic iodine fraction.

The experiment in which 100  $\gamma$  of labeled iodide were injected demonstrates clearly (Fig. 2) that the *goitrous* glands have a much greater than normal capacity for fixing injected iodide without converting it to an organic form.

It becomes clear from Fig. 2 that the effect of such drugs as thiouracil on the *total* uptake of radioiodide by the thyroids depends on the interval elapsing after the injection of the  $I^{131}$ . For example, at the 4 hour interval it might be concluded that the uptake by the *goitrous* thyroids is greater than normal, whereas in 26 hours the reverse conclusion would be drawn. These findings, together with those of Vanderlaan and Bissel (10), serve to resolve at least partially the discrepancies which occur in the reports of those investigators, on the one hand, who find a depressing effect of thio-

uracil-like drugs on  $I^{131}$  uptake by the thyroids (12-15) and those who, on the other hand, have reported increased uptakes in animals treated with thiouracil (8, 9).

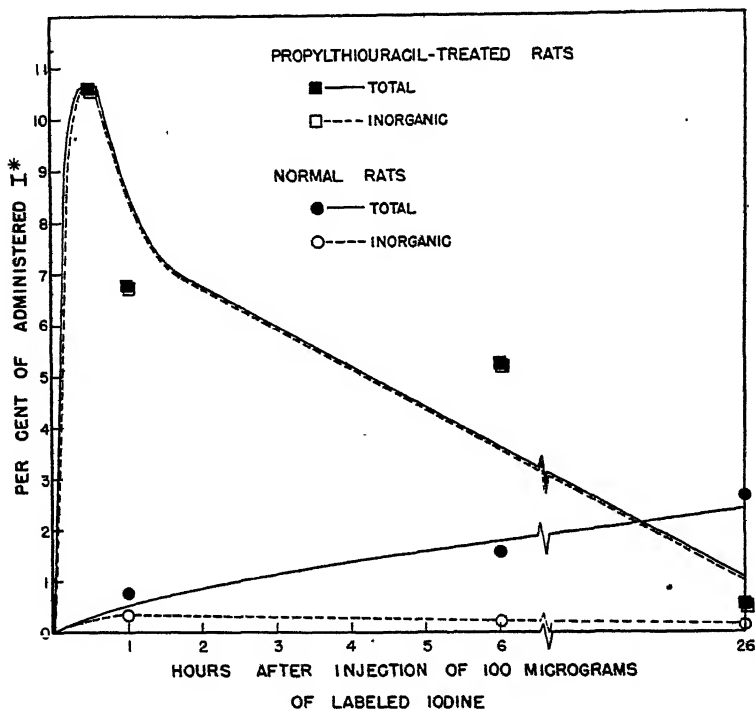


FIG. 2. The uptake of 100  $\gamma$  of labeled iodine by the thyroids of normal and propylthiouracil-treated rats. The latter received a diet containing 0.15 per cent propylthiouracil for 15 days.

*Uptake of 2  $\gamma$  of Labeled Iodine by Glands of Rats Fed  
Propylthiouracil for 12 Hours*

In Table II are shown the results of an experiment in which rats were treated with propylthiouracil for only 1 night and the uptake of radioiodide by their thyroids measured 5 minutes and 5 hours after the injection of 2  $\gamma$  of labeled iodine. The treatment with propylthiouracil was too short to affect the size of the gland. The results at the 5 hour interval indicate, however, that it almost completely blocked the formation of organic iodine.

It should be noted that the total uptake in the first 5 minutes after the  $I^{131}$  injection was not affected by the propylthiouracil treatment. Thus it is demonstrated here that a non-enlarged thyroid gland, which is unable

to synthesize organically bound iodine, retains its capacity to fix iodine initially. This non-organic initial fixation of iodine by an apparently normal thyroid thus appears to be a process which is independent of the subsequent conversion of the iodine to diiodotyrosine and thyroxine.

TABLE II  
*Uptake of 2  $\gamma$  of Labeled Iodide by Thyroids of Rats Fed 0.15 Per Cent Propylthiouracil for 1 Night*

Interval after $I^{131}$ injection	Treatment	Weight of rat gm.	Weight of thyroids mg.	Per cent of injected $I^{131}$ taken up by thyroids			$\frac{\text{Inorganic } I^{131} \times 100}{\text{Total } I^{131}}$
				Total	Inorganic I fraction	Organic I fraction	
<i>min.</i>							
5	Control	283	21	0.47	0.18	0.29	39
5	"	278	26	1.28	0.86	0.44	66
5	"	272	25.5	1.19	0.46	0.73	38
5	"	276	24.5	1.30	0.61	0.68	47
5	Propylthiouracil	245	23	0.77	0.75	0.018	98
5	"	198	21	1.33	1.31	0.021	98
5	"	253	26.5	1.29	1.26	0.027	98
5	"	232	21.5	1.12	1.10	0.014	99
<i>hrs.</i>							
5	Control	215	21	42.2	0.68	41.5	1.6
5	"	218	18.5	40.7	0.70	40.0	1.7
5	"	275	22	37.1	0.71	36.4	1.9
5	Propylthiouracil	224	19.5	2.8	2.5	0.30	89
5	"	227	25	2.8	2.4	0.36	86
5	"	204	20.5	4.1	3.9	0.18	95

*Relation of Iodine Concentration in Thyroid to That in Plasma in Propylthiouracil-Treated Rats*

A group of twelve rats was fed a stock diet containing 0.15 per cent propylthiouracil for 16 days. At the end of this period they were injected intraperitoneally with 100  $\gamma$  of iodide (as KI, not labeled) and sacrificed at the following intervals thereafter, 0.5, 2, 6, and 24 hours. The average concentrations of iodide in plasma and thyroids are plotted in Fig. 3. From the resulting curves it can be seen that the ratio of the thyroid iodide concentration to the plasma iodide concentration remains roughly constant. This ratio has the value 270 in 0.5 hour and 220 in 24 hours. It appears, therefore, that in goitrous rats the thyroid and plasma iodide concentrations are in rapid equilibrium, the values found in these two tissues being related linearly by a constant proportion of approximately 250. This proportion is apparently independent of the amount of iodide

injected, for the two curves obtained for the goitrous thyroids, namely that after the injection of a tracer dose (Fig. 1) and the other after the injection of 100  $\gamma$  of iodide (Fig. 2), are quite similar with respect to (1) the percentage of the administered dose taken up and (2) the time when maximum uptake occurred.

In some of the propylthiouracil-treated animals the concentration of iodine in the liver was compared with that in plasma and in thyroid. The liver showed no iodine-concentrating capacity whatsoever; its iodine concentration was always lower than that of plasma.

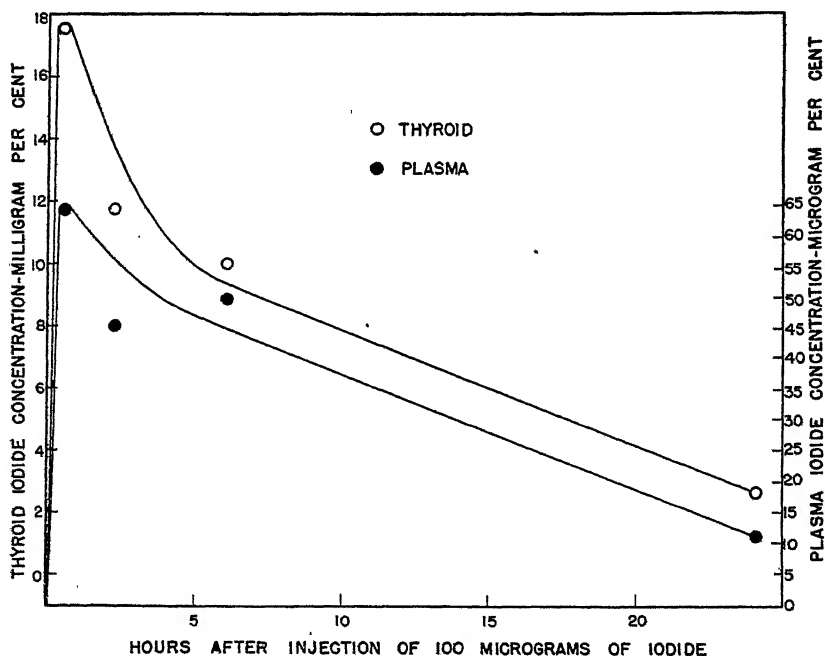


Fig. 3. The concentrations of inorganic iodide in thyroid and plasma of propylthiouracil-treated rats at various intervals after the injection of 100  $\gamma$  of iodide. The rats were fed a diet containing 0.15 per cent propylthiouracil for 16 days.

#### *Chemical Nature of Iodine Which Accumulates in Thyroids of Propylthiouracil-Treated Rats*

*Trichloroacetic Acid Solubility*—It was pointed out above that all the iodine fixed by the enlarged thyroids of propylthiouracil-treated rats appears in the trichloroacetic acid-soluble fraction, whereas in the normal gland all or nearly all of the iodine is precipitated by trichloroacetic acid along with the protein. This finding is in accord with the results of McGinty (8) and of Astwood (9). The former showed that the iodine

taken up by the goitrous glands is not precipitated by treatment with NaOH and ZnSO<sub>4</sub>; the latter showed that practically all of the iodine taken up can be extracted from the oven-dried gland with distilled water. Although these observations indicate that the iodine held by the goitrous glands is not firmly bound to protein, the possibility of a loose protein linkage easily disrupted by manipulating the tissue is not ruled out.

*Behavior toward Iodide Carrier*—The identification of the chemical form in which iodine is fixed by the *goitrous* glands has been greatly facilitated by the use of radioactive iodine. It is shown in the following experiment that the behavior of the radioactive iodine fixed by the thyroids of propylthiouracil-fed rats is identical with that of non-radioactive inorganic iodide added as a carrier during the extraction procedure.

TABLE III

*Nature of the Iodine in Thyroids of Propylthiouracil-Treated Rats Injected with 100  $\gamma$  of Labeled Iodide*

Interval after I <sup>131</sup> injection	Weight of rat	Weight of thyroids	Per cent of injected I <sup>131</sup> taken up by thyroids		
			Whole gland	Trichloroacetic acid-soluble	
				Total	Extractable as I <sub>2</sub> after oxidation with iodate
hrs.	gm.	mg.			
1	266	51	7.6	7.6	7.4
1	180	52	7.0	7.0	7.3
6	188	47	5.3	5.3	5.0
6	235	63	4.7	4.7	4.8
26	185	53	0.68	0.67	0.64

100  $\gamma$  of labeled iodide were injected into rats that had been fed 0.15 per cent propylthiouracil for 14 days. The glands, excised 1, 6, and 26 hours after the injection, were homogenized with 1 cc. of 10 per cent trichloroacetic acid. 2 mg. of iodide carrier were then added to this acid extract of the gland and quantitatively oxidized to I<sub>2</sub> with excess iodate. The I<sub>2</sub> was extracted with carbon tetrachloride and then reextracted with dilute thiosulfate solution. All of the radioactive iodine which had originally been in the trichloroacetic acid extract was recovered in the thiosulfate solution (Table III). This finding indicates that the iodine in the trichloroacetic acid extract of the goitrous gland must have been in the form of either inorganic iodide (I<sup>-</sup>) or iodine (I<sub>2</sub>).<sup>1</sup> Its presence as organic iodine

<sup>1</sup> The radioiodine in the trichloroacetic acid extract of the thyroids of *normal* rats that had been injected with 100  $\gamma$  of labeled iodine was also quantitatively recovered in a thiosulfate solution by the above procedure.

(i.e. C-I bond) is excluded, since the latter does not react with iodate to give  $I_2$  under the conditions used above.

To test the possibility that the thyroid iodine existed in the form of  $I_2$ , thyroids removed from propylthiouracil-treated rats that had been injected with labeled iodide were ground with distilled water or with dilute acid and the resulting homogenate extracted directly with carbon tetrachloride. Only a negligible fraction (less than 1 per cent) of the thyroid  $I^{131}$  appeared in the carbon tetrachloride phase. However, if the acid homogenate was first treated with excess iodate (no iodide carrier), more than 80 per cent of its radioactivity was found in the carbon tetrachloride layer. These

TABLE IV  
*Dialysis and Ultrafiltration Experiments*

See the text for details.

Weight of rat	Weight of thyroid	Per cent of administered $I^{131}$ in thyroids	Treatment of thyroid	Duration of treatment	Per cent of thyroid $I^{131}$ in	
					Dialysate or ultrafiltrate	Residue
gm.	mg.			hrs.		
274	84	13.6	Dialysis of distilled water homogenate of tissue against distilled water	16	100	0.5
246	95	15.1	Dialysis of intact thyroid glands against distilled water	17	99.5	0.5
340	99	11.5	Dialysis of intact thyroid glands against distilled water	5	95.0	5.0
340	99	11.5	Dialysis of intact thyroid glands against oxygenated phosphate buffer, pH 7.3	5	94.5	5.5
306	62	13.4	Ultrafiltration of distilled water homogenate of tissue	16	97.6	2.4

findings justify the conclusion that the iodine which collects in the thyroids of propylthiouracil-treated rats is not in the form of  $I_2$  but rather in the form of iodide ( $I^-$ ).

*Dialysis and Ultrafiltration Experiments*—Rats which had been maintained on 0.15 per cent propylthiouracil for 16 to 19 days were injected with 100  $\gamma$  of labeled iodide and their thyroids removed 0.5 hour later. The goitrous thyroids were homogenized with cold distilled water and transferred to a Visking casing for dialysis or to a collodion bag for ultrafiltration. All experiments were carried out in a cold room maintained at 5–10°.

Each dialysis bag, containing about 5 cc., was attached to a stirrer and dialyzed against 150 cc. of distilled water for 5 to 6 hours. At the end of this period the dialysate was replaced with 100 cc. of fresh distilled water and dialysis continued for another 10 to 12 hours.

Ultrafiltration was carried out by placing a slight positive pressure on the thyroid homogenate contained in the collodion bag, a procedure which required about 12 hours for completion. The clear ultrafiltrate gave a negative test for protein.

The results in Table IV show that under the above conditions practically all the radioiodine contained in the goitrous glands passed through the semipermeable membrane.<sup>2</sup> Complete passage of the radioiodine through the membrane was also observed even when the intact thyroid glands (*i.e.* not ground) were placed within the dialysis bag together with 5 cc. of distilled water or 5 cc. of oxygenated phosphate buffer. The dialysis was almost quantitative in 5 hours, 95 per cent of the radioiodine appearing in 150 cc. of dialysate within this time.

#### DISCUSSION

It was suggested earlier, on the basis of experiments with surviving thyroid slices, that there exists in the thyroid gland a mechanism for the selective uptake of iodine independent of its conversion to diiodotyrosine and thyroxine (6, 7). The existence of such a mechanism is amply supported by the results presented here on the intact thyroid (Figs. 1 to 3). As much as 15 per cent (Table IV) of an injected dose of 100  $\gamma$  of iodide appeared in 30 minutes in the thyroids of propylthiouracil-fed rats despite the fact that the synthesis of organic iodine compound by these glands was almost completely inhibited. At this time interval the iodide concentration in the thyroid was 200 to 300 times that in the plasma. The iodine was not held in the gland for long; practically all of it disappeared within 24 hours. The failure of such glands to retain iodine may be explained by their inability to convert it to the organic form in which it is normally held by the gland.

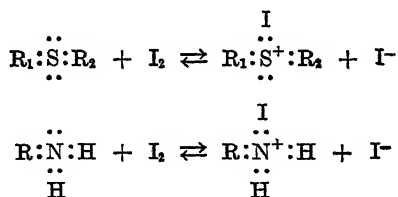
The fact that the gland of the propylthiouracil-treated animal possesses a mechanism for concentrating iodine independent of its conversion to diiodotyrosine and thyroxine suggests, but does not prove, that a similar mechanism exists in the *normal* gland. Its existence in the normal gland, moreover, is rendered plausible by the results of the experiment in which rats were exposed to the goitrogenic diet for *only 12 hours* (Table II); organic iodine formation in their thyroids was completely inhibited, even though the glands remained normal in size. The uptake of  $I^{131}$  by these glands at a very short interval after the injection of the radioiodine (5 minutes) did not differ significantly from that in controls (Table II). These results show that the *initial* fixation of iodine (*i.e.* non-organic) by the thyroid gland was not interfered with. They suggest therefore not

<sup>2</sup> Only 5 per cent of the radioiodine contained in the *normal* thyroid was dialyzable in 24 hours.

only that the normal gland has a mechanism for binding iodine in a non-organic form, but that the *primary* action of such drugs as thiouracil is on the synthesis of organic iodine and not on the iodine-trapping mechanism.

Further evidence for the existence of a non-organic iodine-binding mechanism in the *normal* thyroid is provided by the finding (Fig. 2) that 1 hour after the injection of 100  $\gamma$  of labeled iodide into *normal* rats the concentration of newly fixed *inorganic iodide* in the thyroid gland was at least 1000  $\gamma$  per cent, whereas that of plasma was less than 100  $\gamma$  per cent. It appears from these results that the mechanism in the *normal* thyroid gland for synthesizing organic iodine is more rapidly saturated than the iodide-fixing mechanism of the gland (4).

What is the mechanism by which the thyroids of propylthiouracil-treated rats are able to hold iodine in a non-organic form at a concentration 200 to 300 times as great as that in plasma? It is reasonable to suppose that the iodine fixed by these glands is held in some sort of loose chemical linkage. Such a type of linkage is exemplified by the dihalide addition products which form the basis of Lavine's method for determination of methionine (16). Such compounds (periodides) are formed by reactions between  $I_2$  and thio ethers, amino groups, and other groups possessing a free pair of electrons.



In acid solution the periodides decompose with the liberation of  $I_2$ . The evidence obtained here definitely opposes the view that such linkages play an important rôle in the fixation of iodine by the goitrous glands, since none of the iodine taken up by these glands could be extracted directly with  $\text{CCl}_4$  from an *acidified* homogenate of the tissue. Moreover, only after the addition of an oxidizing agent such as iodate to the homogenate was the fixed iodine readily extracted with  $\text{CCl}_4$ . These findings, together with the observation that all of the iodine taken up by the goitrous glands behaves exactly as does added iodide carrier, may be taken as good evidence that the fixed iodine remains in the gland in the form of inorganic iodide ( $\text{I}^-$ ). A similar conclusion was reached by Vanderlaan and Vanderlaan (17).

Thus, while it is definitely established that the iodine taken up by the goitrous gland is present as  $\text{I}^-$ , the exact nature of the linkage that binds



it is not known. Whatever its nature, this linkage must explain the following experimental findings: (1) The thyroid iodine concentration is in relatively rapid equilibrium with the plasma iodine level but remains about 200 to 300 times as great as the latter. (2) The thyroid iodine does not precipitate with the proteins when the tissue is homogenized with trichloroacetic acid, or with sodium hydroxide and zinc sulfate. (3) The thyroid iodine is completely dialyzable and ultrafiltrable. (4) The uptake of iodine by the goitrous gland is inhibited by KCNS (10).

Salter *et al.* have postulated the existence of what they term "protein-bound inorganic iodide" to explain the iodine-trapping mechanism of the thyroid (18). While the above evidence does not rule out such a concept, it does provide certain limitations to the type of protein-iodide linkage which is possible.

#### SUMMARY

1. Thyroids of rats made goitrous by prolonged feeding of propylthiouracil possess a mechanism for concentrating injected iodine despite a complete block in the formation of organic iodine. This concentrating mechanism can be demonstrated after the injection of a carrier-free dose of radioiodide as well as after the injection of an amount as large as 100  $\gamma$  of iodide. The capacity of these goitrous glands for fixing injected iodide in a non-organic form is much greater than normal.

2. In propylthiouracil-treated rats the curves depicting the changes in the inorganic iodide concentration of plasma and of thyroid tissue after the injection of 100  $\gamma$  of iodide are similar in shape. In both, the maximum concentration is reached within 0.5 hour, the iodide concentration falling rapidly thereafter (Fig. 3). A linear relation was found between the iodide concentrations in goitrous thyroid and plasma, the former at all intervals being 200 to 300 times as great as the latter. The extent to which non-organic iodine trapping can occur in the thyroids of propylthiouracil-treated rats is therefore limited by the concentration of plasma iodide.

3. The following points are established about the chemical nature of the iodine taken up by the *goitrous* glands: (a) It is all in the form of inorganic iodide ( $I^-$ ). (b) It is not stably bound to protein, although the possibility of a very labile protein linkage is not ruled out.

4. In rats treated with propylthiouracil for only 12 hours the thyroids are normal in size but are unable to synthesize organically bound iodine compounds. Such glands, however, show the same *initial* rate of fixation of injected iodide as do the glands of untreated rats. This suggests that even in the *normal* thyroid there is a mechanism for fixing iodine which is independent of its subsequent conversion to diiodotyrosine and thyroxine.

5. The characteristics of the linkage responsible for concentrating iodine in a non-organic form in thyroid tissue are discussed.

*Addendum*—After this manuscript had been submitted for publication there appeared an extensive article by Vanderlaan and Vanderlaan dealing with the same subject (19). Their findings are in good agreement with the data presented here.

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## THE EFFECT OF SOME AMINO ACIDS ON THE GROWTH AND NICOTINIC ACID STORAGE OF RATS ON LOW CASEIN DIETS\*

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Krehl *et al.* (1, 2) have observed that the inclusion of large amounts of corn grits in a low protein diet produced a growth retardation in rats, which was corrected by the addition of either nicotinic acid or L-tryptophan. The beneficial action of the amino acid is now attributed to its rôle as a biological precursor of nicotinic acid (3-5). It is not necessary to postulate the existence of a specific pellagragenic agent in corn for the rat, at least, because the deficiency syndrome with corn can be duplicated with non-corn rations by the addition of tryptophan-deficient proteins or acid-hydrolyzed proteins to a nicotinic acid-deficient diet containing suboptimal amounts of tryptophan (6). Inasmuch as nicotinic acid produced normal growth on the nicotinic acid-deficient diets only when these protein supplements were present, it appears probable that the unsupplemented diet was also inadequate with regard to some amino acids. In this respect, Hall and Sydenstricker (7) have observed normal growth in rats when lysine, valine, threonine, histidine, and tryptophan were added to low casein diets adequate in other respects.

We report here the production of a severe nicotinic acid deficiency in the rat on low protein diets supplemented with some amino acids and the effect of these substances on the storage of nicotinic acid in the liver.

### EXPERIMENTAL

Wistar rats, 21 to 23 days old, were used in these experiments. The basal diet consisted of casein (Labco) 9, sucrose 82, salts (8) 4, L-cystine 0.2, cottonseed oil 3, and cod liver oil 2 parts. Vitamins were incorporated in 100 gm. of diet at the following levels: thiamine 1.0 mg., riboflavin 1.0 mg., pyridoxine 1.0 mg., calcium pantothenate 2.0 mg., choline chloride 200 mg., 2-methyl-1,4-naphthoquinone 0.5 mg., inositol 10 mg., biotin 0.02 mg., and folic acid 0.2 mg.  $\alpha$ -Tocopherol was administered at a

\* Acknowledgment is made of aid from the John and Mary R. Markle Foundation and the United States Public Health Service. Folic acid and biotin were generously contributed by Lederle Laboratories Division, American Cyanamid Company and Merck and Company, Inc., respectively.

level of 1.0 mg. per rat per week. Supplements of amino acids replaced an equal amount of sucrose in the diet.

The nicotinic acid content of liver was determined microbiologically (9) on tissue extracts prepared by autolysis aided by taka-diastase and papain (10).

From the results in Table I it is evident that the growth on the basal ration can be improved to a limited extent by the addition of either nicotinic acid or tryptophan. Even here, however, values remain below the rate of 21 gm. per week of our normal stock animals. The nicotinic acid content of the livers of these animals is similarly increased by supple-

TABLE I  
*Growth and Liver Nicotinic Acid of Rats on Various Diets*

Diet No.	No. of rats	Diet	Gain per wk. for 4 wks.	Liver nicotinic acid
			gm.	$\gamma$ per gm.
1	10	Basal	9 (6-12)*	123 (90-155)*
2	7	" + 10 mg. % nicotinic acid	14 (11-18)	148 (126-173)
3	7	" + 0.2 % L-tryptophan	15 (12-19)	177 (129-227)
4	9	" + amino acid mixture†	2 (-1 to +4)	148 (103-194)
5	9	Diet 4 + 2.0 mg. % nicotinic acid	20 (17-25)	160 (142-180)
6	7	" 4 + 0.2 % L-tryptophan	24 (19-28)	218 (180-276)
7	5	" 4 minus L-lysine	3 (2-3)	126 (100-167)
8	5	" 7 + 2.0 mg. % nicotinic acid	20 (19-21)	172 (149-188)
9	5	" 7 + 0.2 % L-tryptophan	23 (21-25)	209 (182-263)

\* The values in parentheses represent the range.

† Composition of amino acid mixture, L-histidine monohydrochloride 0.25 per cent, L-lysine monohydrochloride 0.52 per cent, DL-valine 0.30 per cent, and DL-threonine 0.40 per cent.

mentary nicotinic acid. With tryptophan, however, normal storage<sup>1</sup> is found, in spite of a suboptimal growth rate.

Lysine, valine, histidine, and threonine, when added to the basal ration to improve the amino acid composition, produce a marked growth depression,<sup>2</sup> which is not reflected in a further decrease in the liver nicotinic acid. In fact, these values are somewhat above those obtained on the basal diet alone. Within this former group the growth rates of -1 to +4 correspond to values of 194 to 103  $\gamma$  for the vitamin content of the liver,

<sup>1</sup> The liver nicotinic acid of our stock animals is 160  $\gamma$  per gm. of tissue.

<sup>2</sup> Most of these animals exhibit a reddish staining of the nose and whiskers and a reddening of the paws, apart from any porphyrin-like accumulation. Corneal vascularization is absent.

indicating that within this group an inverse relationship exists between the growth rate and nicotinic acid storage. The addition of nicotinic acid (Diet 5) or tryptophan (Diet 6) not only corrects the growth depression, but permits a normal growth rate, which is not possible in the absence of these amino acids (Diets 2 and 3). The synergistic effect of nicotinic acid and these amino acids is also reflected in normal nicotinic acid storage in the liver. With supplementary tryptophan, storage values are considerably above normal,<sup>3</sup> indicating perhaps either excessive synthesis or retention of biologically active intermediates in the synthetic process. It is of interest to note that in animals on Diet 3 tryptophan can maintain a normal level of nicotinic acid in the liver, in spite of a subnormal growth.

TABLE II  
*Effect of Acid Hydrolysis on Liver Nicotinic Acid Values*

Diet No.*	Liver nicotinic acid	
	By autolysis	By autolysis + acid hydrolysis
	$\gamma$ per gm.	$\gamma$ per gm.
1	90	101
2	144	139
3	227	226
4	161	174
5	152	154
6	239	232

The values represent data obtained from one litter of six rats. The "autolyzed" data are included in Table I.

\* As given in Table I.

rate, whereas this is not possible if nicotinic acid is preformed in the diet (Diet 2).

Inasmuch as nicotinic acid will promote normal growth when the lysine-deficient protein zein is added to a low protein diet (6), this amino acid was omitted from the amino acid supplement (Diet 7). The effect of this modified amino acid supplement on the growth of rats on the basal diet resembles that observed with the amino acid mixture containing lysine. However, there is no change in the nicotinic acid storage in the liver. With supplementary nicotinic acid or tryptophan the growth depression is corrected and normal growth ensues. It is also evident that the amount of lysine in a 9 per cent casein diet is adequate for normal growth when optimal amounts of other amino acids are present.

<sup>3</sup> In unpublished experiments we have found values of liver nicotinic acid as high as 353  $\gamma$  per gm. in rats on diets containing from 0.5 to 1.0 per cent L-tryptophan.

In a previous communication from this laboratory (5) it was shown that after the administration of tryptophan to rats there was an increased urinary excretion of an unidentified substance, which was converted to nicotinic acid after acid hydrolysis. Apparently this derivative is not present in significant amounts in the livers of rats on various diets in the present work (Table II).

#### DISCUSSION

Sarett and Perlzweig (11) and Wright and Skeggs (12) have shown that the nicotinic acid storage in the liver, regardless of vitamin intake, is directly related to the protein level of the diet. Our results indicate that on a 9 per cent casein diet the addition of nicotinic acid will increase the storage to a limited extent. This restriction of action is apparently related to the existence of deficiencies of some amino acids on the low protein ration, for when these deficiencies are satisfied, dietary nicotinic acid not only permits normal growth, but also normal storage. It is of interest to note in this respect that Salmon (13) observed that nicotinic acid has a more marked growth-promoting action in rats on 12 per cent than on 9 per cent casein rations. It is presumed that at the higher level of casein, with which deficiencies of some amino acids are less evident, nicotinic acid can exert more fully its growth-promoting action.

The production of a severe nicotinic acid deficiency in the rat by the addition of some amino acids to a 9 per cent casein ration may explain the similar deleterious effects of zein, gelatin, or acid hydrolysate of fibrin when added to a low protein ration. Krehl *et al.* (14) have observed that of a number of amino acids tested glycine was particularly effective in depressing growth of rats under similar conditions. In this respect we have found that sodium benzoate does not prevent the growth-inhibitory effect of gelatin in rats on low casein diets.<sup>4</sup> Groschke *et al.* (15) indicate that most amino acids depress growth in chicks on diets low in nicotinic acid. In rat experiments involving the addition of amino acids to a low protein diet, it is well to consider not only the growth-depressing effects of these substances, but also their ability to promote normal growth upon the addition of nicotinic acid.

#### SUMMARY

The growth of rats on a 9 per cent casein diet is improved to a limited extent by supplementary nicotinic acid or L-tryptophan. This is associated with an increased level of liver nicotinic acid. The addition of histidine, valine, threonine, and lysine to the low protein diet produces a

<sup>4</sup> Unpublished data.

marked growth depression, which is not accompanied by a decrease in liver nicotinic acid. The addition of either nicotinic acid or tryptophan not only corrects the growth retardation, but also permits normal growth. When lysine is omitted from the amino acid mixture, essentially similar results are obtained.

No evidence is obtained for the presence in liver of an acid-hydrolyzable derivative of nicotinic acid previously reported present in the urine of rats receiving tryptophan.

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## UTILIZATION OF D-TRYPTOPHAN BY THE CHICK\*

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In a series of investigations on the amino acid requirements of poultry, studies have been carried out on the tryptophan requirement of the chick. It immediately became evident that the rates of gain observed when DL-tryptophan was fed were far greater than could be accounted for on the basis of the amount of L-tryptophan contributed by the DL-tryptophan. These results suggested that the D isomer of tryptophan was utilized to some extent by the chick. The work was extended to obtain additional data. The results obtained with these studies on the utilization of D-tryptophan by the chick are presented in this paper.

### EXPERIMENTAL AND RESULTS

Mixed day-old cross-bred chicks (New Hampshire-white Leghorn) were fed a practical chick starter ration for 10 days. Those individuals that deviated the greatest from the average performance were discarded. Ten chicks were then selected for each experimental group, so that the average gain and weight were the same for all groups. The basal diet used consisted of (in per cent) oxidized casein (1) 12, gelatin 10, mineral mixture (2) 5, corn oil 5, fish solubles 2 (dry basis), L-cystine 0.5, DL-methionine 1.0, fortified cod liver oil 0.75, and corn-starch to 100. The following amounts (in mg.) of vitamins were added to each 100 gm. of diet; thiamine 0.60, riboflavin 0.66, pyridoxine 0.66, calcium pantothenate 2.2, nicotinic acid 5.0, inositol 100, choline 200, *p*-aminobenzoic acid 0.2, biotin 0.02, pteroylglutamic acid 0.2, 2-methyl-1,4-naphthoquinone 2.0, and mixed tocopherols 10.0. The vitamins and graded levels of L- and DL-tryptophan<sup>1</sup> were added at the expense of the starch. When sufficient tryptophan was added to this ration, the rates of growth obtained were comparable to those obtained with rations which contained 12 or 18 per cent untreated casein supplemented with cystine, methionine, and tryptophan.

\* Some of the experimental data have been taken from a thesis submitted by M. C. Wilkening in partial fulfillment of the requirements for a degree of Master of Science in Biochemistry and Nutrition.

<sup>1</sup> We are indebted to The Dow Chemical Company for generous gifts of DL-tryptophan. The L-tryptophan was purchased from General Biochemicals, Inc.

From these results it was apparent that other amino acids were present in adequate quantities in the basal ration.

The groups were fed the tryptophan supplements indicated in Table I for a period of 14 days. It can be seen that the rate of growth obtained with various levels of DL-tryptophan was much greater than that observed for equivalent levels of L-tryptophan. For example, chicks fed 0.1 per cent L-tryptophan gained 20 gm. during the 14 day test period, while chicks fed 0.2 per cent DL-tryptophan gained 72 gm., in spite of the fact that both rations contained the same level of L-tryptophan. The difference in growth rates was shown to be highly significant ( $P < 0.001$ ). In all cases the levels of tryptophan used were not above those required for optimum growth. By microbiological analysis with *Streptococcus faecalis*

TABLE I  
*Activity of D-Tryptophan for New Hampshire-White Leghorn Chicks*

Experiment No.	Quantity of tryptophan added to basal diet	Isomer	Average gain for 2 wk. period	Apparent activity of D-tryptophan	Feed efficiency*
	<i>per cent</i>		<i>gm.</i>	<i>per cent</i>	
1	0.10	L	20		0.13
	0.15	"	112		0.38
	0.15	DL	35	40	0.18
	0.20	"	72	30	0.37
	0.25	"	117	26	0.42
	0.30	"	143	17	0.50
2	0.125	L	27		0.15
	0.20	DL	56	40	0.32

\* Gm. gained per gm. of food consumed.

As the test organism, the DL-tryptophan was 50 per cent as active as the L-tryptophan. The medium used in these tests was the same as that described previously, with the appropriate amino acid omitted (3). Therefore, the results obtained with the chick can be explained on the basis of utilization of the D isomer. For each level of DL-tryptophan fed, the apparent activity of the D isomer was calculated from the growth data obtained with groups fed graded levels of L-tryptophan. The utilization of the D isomer observed for the various groups fed DL-tryptophan ranged from 17 to 40 per cent (Table I). Results obtained on the efficiency of feed utilization were in excellent agreement with those obtained for growth and are also included in Table I.

Recent studies by Briggs and associates (4) show that, with rations containing 10 per cent gelatin, the chick requires approximately 5 mg. of nicotinic acid per 100 gm. of ration. Although the basal ration was

supplemented with 5 mg. of this vitamin per 100 gm. of ration, additional studies were carried out with 10 mg. of nicotinic acid added. The growth rates were identical for groups fed the 0.2 per cent DL-tryptophan ration with 5 or 10 mg. of nicotinic acid per 100 gm. Therefore, it was assumed that sufficient nicotinic acid was added to the basal diet, and the lower level was used in subsequent experiments.

By measurements of feed efficiency, Grau and Almquist (5) concluded that the D isomer of tryptophan was not utilized by the white Leghorn chick. Therefore a series of experiments was devised with mixed white Leghorn chicks to extend the observations made with the New Hampshire-white Leghorn cross. The same experimental regimen was used in this work and ten carefully selected birds were included in each group. The experimental period was terminated after 12 days in order to conform to

TABLE II  
*Activity of D-Tryptophan for White Leghorn Chicks*

Quantity of tryptophan added to basal diet	Isomer	Average gain for 12 day period	Apparent activity of D-tryptophan	Feed efficiency*
<i>per cent</i>		<i>gm.</i>	<i>per cent</i>	
None		-13		
0.10	L	18		0.15
0.135	"	66		0.33
0.175	"	88		0.44
0.15	DL	13	23	0.08
0.30	"	89	17	0.42

\* Gm. gained per gm. of food consumed.

the length of time used by Grau and Almquist. The results are shown in Table II. The utilization of the D isomer of tryptophan obtained with the white Leghorn appeared to be somewhat less than that observed with the cross-bred chicks.

#### DISCUSSION

The basal ration used affords an excellent basis for measurements of the utilization of D-tryptophan as well as of the actual quantitative requirements for tryptophan. The basal ration contributes approximately 0.008 per cent of L-tryptophan and the cross-bred chicks fed this ration lost an average of 18 gm. in the 2 week test period. Birds fed an optimum level of tryptophan, however, gained an average of 144 gm. in this period. Thus, a rather extensive range of gain was utilized to measure the activity of D-tryptophan at several levels. Similarly, the feed efficiency computed as the average for each group of ten birds showed a similar range. The

results obtained with all levels of DL-tryptophan below the amount needed for maximum growth demonstrate activity for the D isomer ranging from 17 to 40 per cent.

No obvious explanation is available for the discrepancies in these results and those of Grau and Almquist. It is quite possible that the inclusion of pteroylglutamic acid or higher levels of nicotinic acid or other variants in the dietary and experimental conditions may be contributing factors. These possibilities afford suggestions as to the factor or factors which may play a rôle in the ability of an organism to convert effectively the unnatural isomer to the natural and biologically active isomer.

The experimental techniques have also been employed to evaluate the quantitative requirement of the chick for tryptophan. These data will be presented in a separate paper.

#### SUMMARY

1. A ration in which the major portion of the protein is supplied by oxidized casein and gelatin was devised to study the utilization of D-tryptophan by the chick.

2. New Hampshire-white Leghorn and white Leghorn chicks were used and the utilization of D-tryptophan was evaluated on the basis of both rate of gain and efficiency of feed utilization.

3. The results show that D-tryptophan is utilized to an extent of 17 to 40 per cent. The percentage of utilization appeared to be slightly higher at lower levels of the test compound.

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# THE OXIDATION OF LACTOSE AND MALTOSE TO BIONIC ACIDS BY PSEUDOMONAS\*

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Studies in this Laboratory on the biochemical activities of the genus *Pseudomonas* have led to the isolation of gluconic and 2-ketogluconic acids (1),  $\alpha$ -ketoglutaric acid (2), and the pentonic acids (3) as oxidation products of various monosaccharides. The analogous production of bionic acids from reducing disaccharides by oxidation of the free aldehyde group to a carboxyl group seemed to be a logical extension of these studies.

The difficulty in isolating and characterizing the very water-soluble bionic acids has undoubtedly retarded investigation of them. Gluconic acid is readily obtained in the pure state by crystallization of its calcium salt; no such simple procedure is available in the case of the bionic acids. The only known crystalline metallic salt of a bionic acid, calcium lactobionate, precipitates in such a gelatinous form as to be useless for purification.

The bionic acids were first prepared by Fischer and Meyer (4) as early as 1889, but only in recent years have they received extensive study. A number of attempts (5-7) have been made to increase the low yields obtained by bromine water oxidation; these culminated in the electrolytic method of Isbell and Frush (8) at the National Bureau of Standards. In addition, Isbell prepared for the first time the crystalline lactobionic  $\delta$ -lactone (9) and studied the preparation and properties of the crystalline double salt of calcium bromide and calcium lactobionate (10).

The biochemical production of bionic acids, on the other hand, has not been referred to in the literature. The oxidizing action of *Pseudomonas* on reducing disaccharides has, however, been studied by Lembke (11) who made preliminary manometric measurements of oxygen uptake by lactose and maltose. It was found that lactose was oxidized to about the same extent as glucose by *Bacterium pyocyaneum*, *B. fluorescens*, and *B. syn-cyaneum*, while *B. putidum* was without action on lactose. These four species showed little or no oxygen uptake with maltose. No attempt

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appears to have been made by Lembke to isolate or identify oxidation products.

Investigation of the formation of bionic acids by microorganisms has no doubt been discouraged by the wide-spread ability of microorganisms to cleave enzymatically the glucosidic linkage of the reducing disaccharides. In fact, the question of whether or not any microorganisms can metabolize reducing disaccharides without prior hydrolysis has been a subject of controversy. It was, therefore, one of the objectives of our work to seek a definite answer to this question, at least for some members of the genus *Pseudomonas*, by attempting the isolation of the bionic acids.

In our oxidation studies we examined fifteen species of *Pseudomonas* and found that *P. graveolens* was capable of oxidizing lactose to calcium lactobionate, in rotating drums, with a yield of 75 per cent in 165 hours. Some of the species (*P. putida*, *P. mucidolens*, *P. myxogenes*, *P. aeruginosa* (Vendrell strain), *P. pavonacea*, *P. putrefaciens*, *P. fluorescens*, *P. chlororaphis*, and *P. syncyanea*) were also able to oxidize lactose to an acid in considerable amount. The fermentation time, however, was so long that this group of microorganisms was not studied further nor was the nature of the oxidation products determined. The remaining species (*P. ovalis*, *P. mildenbergii*, *P. synxantha*, *Chromobacterium iodinum*, and *P. saccharophila*) were almost without action on lactose.

With maltose, *Pseudomonas graveolens* again proved to be the best of the eighteen species tested for bionic acid production. A 77 per cent yield of calcium maltobionate was obtained in 50 hours in a rotating drum. *P. fragi* also oxidized maltose at a good rate, but the high calcium values suggested oxidation beyond the bionic acid stage. The remaining species (*P. putida*, *P. mucidolens*, *P. myxogenes*, *P. mildenbergii*, *P. aeruginosa* (Vendrell strain), *P. pavonacea*, *P. fluorescens*, *P. boreopolis*, *P. syncyanea*, *P. ovalis*, *P. schuylkilliensis*, *P. synxantha*, *Chromobacterium iodinum*, *P. putrefaciens*, *P. saccharophila*, and *P. chlororaphis*) oxidized maltose too slowly to be of interest for the preparation of the bionic acids. The first nine species of this group were also found to hydrolyze maltose.

The crude calcium lactobionate was obtained by concentration of the filtered culture liquor to dryness. The salt was a white, non-hygroscopic, amorphous powder showing analytical figures in fair agreement with those for calcium lactobionate. This crude calcium salt from the fermentation could be readily purified by means of the difficultly soluble basic calcium salt.

The identity of the calcium lactobionate obtained by fermentation was established by hydrolysis to D-galactose and calcium D-gluconate. The galactose, which was recovered in 85 per cent yield, was characterized by its melting point, rotation, x-ray diffraction pattern, and by conversion to

the characteristic *o*-tolyl hydrazone. The calcium gluconate, isolated in 86 per cent yield, was characterized by its rotation and x-ray diffraction pattern.

Further confirmation of the identity of the lactobionic acid was supplied by conversion of the crude calcium salt into the crystalline double salt of calcium bromide and calcium lactobionate. This double salt gave an x-ray diffraction pattern identical with that of a synthetic sample kindly supplied by Dr. H. S. Isbell of the National Bureau of Standards. Crude and purified calcium maltobionate samples were obtained in the manner already described for calcium lactobionate. The identity of the natural maltobionic acid was established by comparison of its brucine salt with the corresponding salt of synthetic maltobionic acid prepared by the electrolytic method.

These preliminary studies indicate that the biochemical production of bionic acids in quantity, in good yield, and in high purity can easily be accomplished by using the proper strain of *Pseudomonas*. It has been established, thereby, that at least some microorganisms can metabolize disaccharides without prior hydrolysis.

### *Materials and Methods*

*Preliminary Fermentation Studies*—To find species of sufficient interest to warrant larger scale studies, fermentations were carried out with 100 ml. of solution. The culture solution was contained in Pyrex tubes incubated at 30° and fitted with finely porous stones through which air flowed at the rate of 100 ml. per minute.

The inoculum for each of the 100 ml. cultures was grown for 24 hours in 8 ml. of a liver extract medium which initially contained 0.2 per cent of glucose. The fermentation medium contained, in addition to approximately 100 gm. of disaccharide, 0.6 gm. of  $\text{KH}_2\text{PO}_4$ , 0.25 gm. of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , and 5 ml. of corn steep liquor per liter. 3 drops of soy bean oil were added to each culture as an antifoam agent. At the time of inoculation, 1 ml. of sterile 20 per cent urea solution and 2.5 gm. of  $\text{CaCO}_3$  (sterilized dry) were added to each culture.

*Drum Runs*—The rotating drums were those described by Herrick, Hellbach, and May (12). For the 3 liters of fermentation liquor, a 100 ml. culture such as was used for the preliminary studies served as inoculum. Nutrients were supplied to the drum cultures in the same concentrations as to the test-tube cultures. The air flow through the 3 liter cultures was 1200 ml. per minute, the temperature was 25°, and the rate of rotation was 9.5 R.P.M.

Calcium was determined on filtered culture liquors by permanganate titration after oxalate precipitation. Maltose and lactose were determined



by the method of Shaffer and Hartmann (13) by use of specially prepared curves for the copper-sugar ratios.

Culture numbers appearing in the experimental part are those of the Culture Collection, Fermentation Division, Northern Regional Research Laboratory, Peoria, Illinois.

#### EXPERIMENTAL

##### *Studies on Lactose*

*Preliminary Fermentations*—100 ml. cultures containing 9.60 gm. of lactose were fermented with fifteen different species of *Pseudomonas*, and samples were removed periodically for sugar determinations. After 6 days of incubation, the *P. graveolens* 14 fermentation was harvested. There was no residual lactose. The other cultures were harvested after 13 days, at which time all gave positive tests for reducing substances with the Shaffer-Hartmann reagent and negative tests for monosaccharides with Barfoed's reagent. Nine of the species (*P. putida* 13, *P. aeruginosa* (Vendrell strain) 23, *P. mucidolens* 16, *P. myxogenes* 19, *P. pavonacea* 24, *P. putrifaciens* 77, *P. fluorescens* 334, *P. chlororaphis* 560, and *P. synzyanea* 652) showed some dissolved calcium, in some cases a considerable amount. The remaining five strains (*P. ovalis* 8, *P. mildenbergii* 21, *P. synzantha* 79, *Chromobacterium iodinum* 141, and *P. saccharophila* 628) consumed almost no lactose.

*Drum Run on Pseudomonas graveolens* 14—3 liters of culture solution, which contained initially 9.30 per cent lactose (anhydrous), were inoculated with a 100 ml. culture of *P. graveolens* 14. After 165 hours the culture contained no residual sugar. Lyophilization of 100 ml. of the filtered culture liquor gave a white powder which, after drying to constant weight at 100° (3.5 hours at 1 mm.), weighed 7.94 gm.  $[\alpha]_D^{25} = +23.8^\circ$  (c 5.1; H<sub>2</sub>O). The analytical values approach those of calcium lactobionate.

Analysis—C<sub>24</sub>H<sub>42</sub>O<sub>24</sub>Ca (754.6). Calculated. C 38.2, H 5.61, Ca 5.31

Found. " 38.4, " 5.95, " 5.10

Based on the calcium analysis, 96 per cent of this crude product was calcium lactobionate, which corresponds to a 75 per cent yield for the fermentation. Hydrolysis of 79.6 mg. of the anhydrous salt gave 38.8 mg. of galactose (calculated for pure calcium lactobionate, 38.0 mg.) as estimated by the Shaffer-Hartmann method. The hydrolysis conditions (0.2 N HCl for 30 minutes at 15 pounds autoclave pressure) were established with calcium lactobionate prepared by the electrolytic method of Isbell and Frush (8). The method gave values accurate to  $\pm 3$  per cent.

*Hydrolysis of Natural Calcium Lactobionate to Galactose and Calcium Gluconate*—The lactobionic acid obtained by means of an ion exchange

resin from 3.02 gm. of the crude salt was heated on the steam bath for 4 hours with 1 N HCl. The cooled hydrolysis mixture was freed of chloride ion with  $\text{Ag}_2\text{CO}_3$  and the excess of silver removed with  $\text{H}_2\text{S}$ . A slight excess of  $\text{CaCO}_3$  was added and the filtered solution concentrated to 10 ml. Addition of 23 ml. of hot methanol precipitated a gum which was washed three times with 15 ml. portions of hot methanol. The gum was dried *in vacuo* to 1.64 gm. of white powder, from which 1.42 gm. of calcium gluconate were obtained by crystallization from aqueous ethanol. The product showed the correct x-ray pattern and rotation ( $+9.3^\circ$ ;  $c$  2.9;  $\text{H}_2\text{O}$ ) for that salt. Based on 96 per cent purity of the crude calcium salt, the isolation of 1.42 gm. of calcium gluconate represented an 86 per cent recovery for the hydrolysis.

The combined methanol extracts from the precipitated gum were concentrated to a syrup which crystallized after standing for several days. Filtration gave 1.17 gm. of white crystals which were shown by their melting point of  $156\text{--}159^\circ$ , their melting point on admixture with pure D-galactose of melting point  $164^\circ$ , their x-ray diffraction pattern, and their rotation ( $[\alpha]_D^{25} = +79^\circ$ ) to be somewhat impure D-galactose. The D-galactose was characterized by conversion in 76 per cent yield to the o-tolyl hydrazone, the identity of which was established by a mixed melting point test with an authentic sample. Based on 96 per cent purity of the crude calcium salt, the isolation of 1.17 gm. of galactose represented an 85 per cent recovery of galactose.

*Isolation of Calcium Bromide Double Salt*—The crude calcium salt from the fermentation (782 mg.) was dissolved in 0.51 ml. of water containing 200 mg. of  $\text{CaBr}_2$ . By seeding with a few crystals of the double salt, obtained through the courtesy of Dr. Isbell, a heavy deposit of fine crystals was obtained. Addition of 50 per cent alcohol and filtration yielded 230 mg. of white crystals showing an x-ray pattern identical with that of the synthetic product of Isbell. The natural product showed a calcium content of 7.27 per cent; the calculated value for  $\text{Ca}(\text{C}_{12}\text{H}_{21}\text{O}_{12})_2 \cdot \text{CaBr}_2 \cdot 6\text{H}_2\text{O}$  is 7.54 per cent.

*Isolation of Pure Calcium Lactobionate from Crude Fermentation Product*—For purification, 22.5 gm. of crude fermentation product were converted to the insoluble basic calcium salt (14). After treatment with carbon dioxide, removal of the calcium carbonate, and lyophilization, 14.7 gm. of a white powder were obtained. It gave good carbon and hydrogen figures for calcium lactobionate, but the calcium value was 0.4 per cent high, owing to inorganic salts. To remove these, the salt was dissolved in 45 ml. of water and 25 ml. of absolute alcohol added dropwise until the solution became cloudy. The amorphous precipitate that slowly settled out was filtered off and the filtrate treated with more alcohol to precipitate a

gum. Trituration with alcohol converted this gum to a granular powder (12.7 gm.). This salt, however, was found by carbon, hydrogen, and ethoxyl determinations to retain a molecule of alcohol even after 2 hours drying at  $98^{\circ}$  *in vacuo*. This alcohol was removed by dissolving the salt in water and lyophilizing. On analysis the results corresponded with those for calcium lactobionate after the compound was dried for 2 hours at  $100^{\circ}$  *in vacuo*.  $[\alpha]_D^{25} = +25.1^{\circ}$  (c 5.2;  $H_2O$ ).

*Analysis*— $C_{24}H_{42}O_{24}Ca$  (754.6). Calculated. C 38.2, H 5.61, Ca 5.31  
Found. " 38.1, " 5.58, " 5.38

Our synthetic calcium lactobionate showed  $[\alpha]_D^{25} = +23.8^{\circ}$  (c 5.9;  $H_2O$ ). We have been unable to find any report in the literature on the rotation of this salt.

### *Studies on Maltose*

*Preliminary Fermentations*—In the same manner already described for lactose, eighteen strains of *Pseudomonas* were grown in 100 ml. of culture liquor. After 4 days the culture of *P. graveolens* 14 had completely oxidized the maltose and was harvested. On the 12th day, the *P. fragi* culture showed no more reducing value and was worked up. The remaining cultures were harvested on the 13th day, although there was residual reducing material present in all cases. Of this group, nine strains (*P. putida* 13, *P. mucidolens* 16, *P. myxogenes* 19, *P. mildenbergii* 21, *P. aeruginosa* (Vendrell strain) 23, *P. pavonacea* 24, *P. fluorescens* 334, *P. boreopolis* 550, and *P. syncyanea* 652) gave good positive tests for monosaccharides with Barfoed's reagent, indicating hydrolysis of the maltose. Either glucose or 2-ketogluconic acid gives positive reactions in this test. The remaining seven strains (*P. ovalis* 8, *P. schuylkilliensis* 9, *P. putrefaciens* 77, *P. synxantha* 79, *Chromobacterium iodinum* 141, *P. chlororaphis* 560, and *P. saccharophila* 628) failed to give positive Barfoed's tests at time of harvest. There was considerable soluble calcium in the cultures of *P. ovalis* 8, *P. schuylkilliensis* 9, *P. mucidolens* 16, *P. myxogenes* 19, *Chromobacterium iodinum* 141, *P. boreopolis* 550, and *P. syncyanea* 652.

As a result of this preliminary work, *Pseudomonas graveolens* 14 and *P. fragi* 25 were selected for further study in the rotating drums.

*Drum Run on Pseudomonas graveolens 14*—In the manner already described for lactose, *P. graveolens* 14 was grown on 3 liters of culture solution containing 8.90 per cent of maltose. After 50 hours the culture solution contained less than 0.3 per cent of maltose and was harvested. Lyophilization of 100 ml. of the filtered culture liquor gave a white powder which, after drying to constant weight at  $100^{\circ}$  (3.5 hours at 1 mm.), weighed

8.26 gm.  $[\alpha]_D^{25} = +105^\circ$  (c 6.0; H<sub>2</sub>O) (Glattfeld and Hanke (15),  $+98.3^\circ$ ). It contained 2.63 per cent of maltose.

Analysis—C<sub>24</sub>H<sub>42</sub>O<sub>24</sub>Ca (754.6). Calculated. C 38.2, H 5.61, Ca 5.31  
Found. " 38.0, " 5.55, " 4.83

Based on the calcium analysis, 91 per cent of this crude product was calcium maltobionate, which represents a 77 per cent yield for the fermentation. Hydrolysis of 89.7 mg. of the crude anhydrous calcium salt gave 41.0 mg. of glucose compared to a calculated value of 42.2 mg. for calcium maltobionate containing 2.63 per cent maltose. The hydrolysis conditions (0.2 N HCl for 30 minutes at 15 pounds autoclave pressure) were established with pure barium maltobionate ( $[\alpha]_D^{25} = +88.2^\circ$ ; c 6.1; H<sub>2</sub>O) prepared by the electrolytic method. The accuracy of the method was  $\pm 3$  per cent.

*Brucine Salt of Natural Maltobionic Acid*—The identity of the natural maltobionic acid was established by comparison of its brucine salt with the corresponding salt of synthetic maltobionic acid. The synthetic brucine salt was prepared as follows: 2 gm. of barium maltobionate were freed of barium with an ion exchange resin and the lyophilized residue warmed on a steam bath with 10 ml. of water and 1.53 gm. of brucine until a clear solution resulted. After filtration and lyophilization, the remaining 2.72 gm. of white powder were dissolved in 2.7 ml. of water, and absolute alcohol (15 ml.) was added almost to the point of cloudiness. Compact bars came down slowly and were filtered off after 6 days. Yield, 960 mg. Dried in air, the product melted at 84–86°. On analysis it proved to be a pentahydrate, as did a number of other samples prepared in the same way.

Analysis—C<sub>35</sub>H<sub>48</sub>O<sub>18</sub>N<sub>4</sub>·5H<sub>2</sub>O (842.8). Calculated. C 49.9, H 6.94, N 3.32  
Found. " 50.0, " 7.23, " 3.42 (Dumas)

Drying at 56° to constant weight gave the anhydrous product of melting point 155–157° (evolution of gas) described by Glattfeld and Hanke (15).

Analysis—C<sub>35</sub>H<sub>48</sub>O<sub>18</sub>N<sub>4</sub> (752.8). Calculated. C 55.8, H 6.43, N 3.72  
Found. " 55.7, " 6.18, " 3.73 (Dumas)

The brucine salt of the natural maltobionic acid was prepared in the manner just described for the synthetic salt. 1 gm. of the crude calcium salt gave 510 mg. of crystalline brucine salt. Recrystallization of 460 mg. of this product gave 240 mg. of pure pentahydrate of melting point 85–87°.

Analysis—C<sub>35</sub>H<sub>48</sub>O<sub>18</sub>N<sub>4</sub>·5H<sub>2</sub>O (842.8). Calculated. C 49.9, H 6.94, N 3.32  
Found. " 50.2, " 6.90, " 3.41 (Dumas)

This material gave the same x-ray pattern as the synthetic pentahydrate. On drying to constant weight, the anhydrous product of melting point 152–154° (evolution of gas) was obtained. Exposure of the dried sample to air overnight resulted in the formation of the pentahydrate (m.p. 84–86°).

*Isolation of Calcium Maltobionate from Crude Fermentation Product*—The crude fermentation product was converted to the insoluble basic calcium salt. After treatment with carbon dioxide, removal of the calcium carbonate, and lyophilization, a product was obtained which gave a high value for calcium. For further purification the salt was precipitated from water by absolute alcohol. Since the product so obtained retained alcohol very tenaciously, as in the case of calcium lactobionate, it was dissolved in water and the solution lyophilized. The analytical figures on this product after drying at 100° for 2 hours *in vacuo* were as follows:

Analysis— $C_{24}H_{42}O_{12}Ca$ (754.6).	Calculated.	C 38.2, H 5.61, Ca 5.31
	Found.	" 38.1, " 5.61, " 5.58

The rotation was  $[\alpha]_D^{25} = +103^\circ$  (c 6.1;  $H_2O$ ).

*Drum Run on Pseudomonas fragi 25*—3 liters of solution which contained 9.2 per cent maltose were oxidized by *P. fragi* 25. After 94 hours only 0.2 per cent of maltose remained and the culture was harvested. Glucose determinations on the culture liquor after hydrolysis showed 3.24 per cent glucose, which is equivalent to 6.2 per cent maltobionic acid. This amount of acid would require a calcium content of 0.33 per cent for the culture liquor; the value 0.47 per cent found indicates the presence of lower molecular weight acids.

#### SUMMARY

A strain of *Pseudomonas graveolens* has been found capable of oxidizing lactose and maltose to the corresponding bionic acids. These acids were isolated as the calcium salts in yields of about 75 per cent and with a purity of the crude fermentation product exceeding 90 per cent. This provides a clear cut example of the oxidation of reducing disaccharides by a microorganism without prior hydrolysis.

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STUDIES ON PROTEINS FROM BOVINE COLOSTRUM  
I. ELECTROPHORETIC STUDIES ON THE BLOOD SERUM PROTEINS  
OF COLOSTRUM-FREE CALVES AND OF CALVES FED  
COLOSTRUM AT VARIOUS AGES\*

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Ehrlich (1) concluded that colostrum from immune mice transmits specific antibodies that are absorbed by the nursing. Howe (2) found that the blood serum of the new-born calf acquires specific protein fractions upon the ingestion of colostrum. If the calf failed to receive colostrum, these fractions did not immediately appear in the blood. These observations have been confirmed by the electrophoretic technique (3, 4).

Calves have been successfully raised from birth on colostrum-free rations in this laboratory (5). Because of the importance of colostrum to the calf (6), the question was raised as to the blood protein distribution in these animals as compared to the normal animal which had received colostrum at birth from the dam. Electrophoretic studies are reported here on the blood serum proteins of normal calves which had been given colostrum and of calves raised without receiving colostrum. The effect on the blood serum proteins of feeding colostrum and a globulin isolated from colostrum to some of these animals at various ages is also included.

EXPERIMENTAL

The experimental calves were acquired from *Brucella abortus*-free herds, and only those calves which were known not to have received colostrum were used for experiment. To insure this, the calves were removed from their dams immediately following parturition and fed the experimental ration as soon as possible (within 12 hours). The calves were fed skim milk (12 pounds of milk per 100 pounds of body weight), 25,000 I.U. of vitamin A, and 250 mg. of ascorbic acid daily. When colostrum was fed, it was taken from the dam whenever feasible and fed to the calf at the rate of 5 pounds of colostrum per 100 pounds of calf. On this diet,

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difficulty from diarrhea was occasionally encountered. When this condition was accompanied by an increase in body temperature, sulfathiazole was administered at a level of 4 gm. the 1st day, and thereafter as indicated by the condition of the calf. When colostrum was fed, the calves were bled immediately prior to and 24 hours after the ingestion of colostrum.

Blood samples were taken from the jugular vein, allowed to clot at room temperature, and refrigerated at 4° for 12 hours, when the serum was removed by centrifugation. The electrophoretic analyses were carried out at pH 8.6 and an ionic strength of 0.088 in barbiturate-citrate buffer (7). The samples were dialyzed against two changes of the buffer for a total of 48 hours at 0°. The electrophoretic analyses were conducted at a potential gradient of 6 volts per cm. at a temperature of 1° in a single section cell, with a modified Tiselius apparatus. The duration of the experiment was usually 2 hours. A schlieren cylindrical lens in conjunction with a diagonal knife edge was used to photograph the boundary. The areas under the curves were measured with a planimeter from an enlarged tracing of the photograph. Measurements are recorded here for the descending side only. The designations used by Tiselius were employed to describe the various protein fractions.

### *Results*

About 90 per cent of the Holstein calves were successfully raised on this ration, as reported previously (5). An abnormally soft feces was encountered with calves fed the skim milk diet. Furthermore, the gain in body weight was less in comparison to calves fed colostrum and whole milk. Because of seasonal and individual variation in the calves, it is difficult to compare the general health with that of more normal animals.

A typical electrophoresis pattern of the serum of a new-born calf before and 24 hours after the ingestion of colostrum is presented in Fig. 1. It is recognized that the several fractions are complex and, in this study, no attempt has been made to secure further resolution. In all patterns of the serum from new-born calves, there was a small but consistent amount (about 2.5 per cent of the total proteins) of protein with a mobility between  $-2.8$  and  $-3.5 \times 10^{-5}$  sq. cm. per volt per second, which area is usually occupied by the plasma fibrinogen. Since this area was masked by the broad  $\gamma$ -globulin peak, it was arbitrarily included as  $\gamma$ -globulin.

In calves fed the skim milk diet only two characteristic changes were evident (Table I). The initially low serum  $\gamma$ -globulin fraction slowly increased over the 8 week period studied. Concurrent with this increase, there was a decrease in the initially high  $\alpha$ -globulin fraction. The  $\beta$ -globulin and albumin fractions showed no consistent general change. The mobilities of the various proteins as calculated in reference to the salt

boundary appeared to decrease slightly during the period of observation. The significance of this finding is not readily apparent. Jameson *et al.*

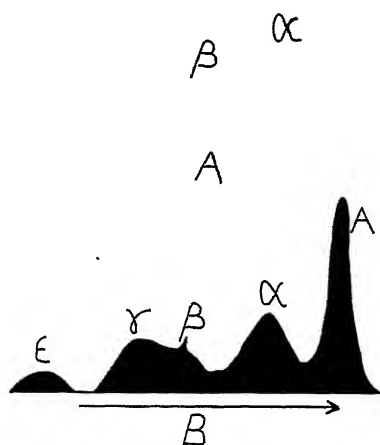


FIG. 1. Electrophoresis patterns of new-born calf serum prior to (A), and 24 hours after (B), the ingestion of colostrum. The experiments were conducted for 120 minutes in a barbiturate-citrate buffer at pH 8.6.

TABLE I  
*Distribution and Mobility of Blood Serum Proteins of Calves Fed Skim Milk*

No. of analyses	Age	Albumin		$\alpha$ -Globulin		$\beta$ -Globulin		$\gamma$ -Globulin	
		per cent*	$\mu$ †	per cent	$\mu$	per cent	$\mu$	per cent	$\mu$
15	Birth	45.4	8.2	37.6	6.1	14.9	4.2	2.8	3.1
1	1 wk.	45.5	8.5	35.0	6.3	15.1	4.3	4.5	2.9
15	2 wks.	47	8.2	30.2	6.1	17.9	4.1	4.8	2.6
2	3 "	50.6	8.1	30.7	6.0	12.8	4.1	5.9	2.6
3	4 "	46.1	7.9	30.5	5.9	14.0	4.0	9.2	2.5
3	6 "	48.9	7.8	26.7	5.8	13.8	4.1	10.4	2.3
3	8 "	45.9	7.8	22.9	5.8	13.6	4.0	18.0	2.4
1	2 yrs.‡	47.4	7.9	16.9	6.1	10.8	3.9	24.6	2.5

\* The area of the salt boundary was neglected in these calculations.

† Calculated from the salt boundary and expressed in sq. cm. per volt per second  $\times 10^{-5}$ .

‡ Normal heifer.

(3) have reported a variation in mobilities of the serum proteins of calves fed colostrum.

Colostrum and a globulin isolated from colostrum were also fed to calves

of various ages which had been raised on skim milk. In no case was there an increase in the  $\gamma$ -globulin fraction (Table II) upon the ingestion of colostrum after the calf was more than 24 hours of age. A water-soluble globulin from colostrum was isolated according to the method of Smith (8), dissolved in skim milk at 38°, and given to calves at ages and at levels indicated in Table II. The resulting increase in the serum  $\gamma$ -globulins was

TABLE II

*Blood Serum Protein Changes in Calves Fed Colostrum or Colostrum Pseudoglobulin*

Treatment	Age	Calf No.	Albu- min	$\alpha$ -Glob- ulin	$\beta$ -Glob- ulin	$\gamma$ -Glob- ulin	
			<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
Colostrum	New-born	77	37.8	28.9	11.1	22.2	After
		88	51.4	40.2	6.7	1.5	Before
			40.8	32	7.6	19.6	After
		89	46.5	41.8	10.6	1.1	Before
			35.7	27.9	12.2	24.0	After
	24 hrs.	90	48.5	41	10.9	1.2	Before
			41.2	35.7	12.2	10.7	After
		87	46.6	30.9	12.7	9.7	"
	42 "	85	46.2	36.2	15.0	2.5	"
			43.0	36.1	14.7	6.1	" *
		86	44.0	35.7	17.5	2.7	"
	1 wk.	76	45.5	35.0	15.1	4.5	Before†
		48	35.2	12.7	4.2		After†
	3 wks.	74	50.3	29.7	12.4	7.4	Before
			52.7	26.4	15.3	5.6	After
			45.3	38.8	12.6	3.4	Before. 40 gm. glob- ulin
Colostrum globulin	New-born	102	45.0	36.8	11.3	7.6	After
		51	43.5	37.8	13.3	4.8	Before. 70 gm. glob- ulin
		39	30.7	18.7	11.5		After
	3 wks.	47	53.3	24.8	14.2	7.8	Before. 70 gm. glob- ulin
			51.6	25.7	16.4	6.4	After

\* 2 weeks after treatment.

† Same colostrum as was fed to Calf 77.

clearly evident in the new-born calves. In contrast no appreciable difference was noted in the blood serum proteins after the administration of 70 gm. of this material to an older animal.

#### DISCUSSION

Difficulty was encountered by early workers (6) in raising calves without feeding them colostrum. Orcutt and Howe (9) demonstrated that an increase in certain blood serum globulins and antibody titer occurred on

the ingestion of colostrum by new-born calves. Smith (10) fed immune serum to two calves, 2 $\frac{1}{4}$  and 18 days old respectively, and found no increase in serum agglutinins or protective antibodies following the ingestion of this material. With calves more than 24 hours of age, we observed no effect, as measured by electrophoresis, on the serum proteins, from the ingestion of colostrum.

Pedersen (11) has reported the isolation of a globulin "fetuin" from bovine fetal blood. This globulin was found present in high concentrations in the serum of new-born calves, although in the serum of the adult animal very little fetuin was observed. Pedersen concluded that fetuin was probably an  $\alpha$ -globulin. Our results are in agreement with this possibility. In the calves raised without colostrum, it is interesting to note the decrease in the  $\alpha$ -globulins of the serum and concurrent with this an appearance of proteins having a mobility in the  $\gamma$ -globulin range. A possible relationship between these two processes is thereby suggested.

#### SUMMARY

Electrophoretic studies have been made on the blood serum proteins of colostrum-free calves and of calves at various ages fed colostrum or a colostrum pseudoglobulin preparation.

There was an immediate increase in the blood serum  $\gamma$ -globulins of calves following the ingestion of colostrum or the colostrum pseudoglobulin during the first 24 hours of life. If these materials were fed to calves after they had reached 24 hours of age, there was no measurable increase in these serum proteins.

When calves were raised without access to colostrum, the various blood serum protein fractions did not approach normal values until the animals were about 8 weeks of age. During this period there was a gradual increase in the serum  $\gamma$ -globulins and a concurrent decrease in the serum  $\alpha$ -globulins in the blood of these calves.

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# STUDIES ON PROTEINS FROM BOVINE COLOSTRUM

## II. SOME AMINO ACID ANALYSES OF A PURIFIED COLOSTRUM PSEUDOGLOBULIN\*

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(Received for publication, June 19, 1947)

Smith (1) has reported the preparation of pseudo- and euglobulins from bovine colostrum, employing ammonium sulfate as a precipitating agent. Since these proteins are recognized to be important in the diet of the young calf, we have isolated them according to the method of Smith and have determined the homogeneity of the products by ultracentrifugation and electrophoresis. The amino acid composition of the pseudoglobulin has been determined. The results of these studies are here reported.

### *Amino Acid Assays*

The protein was hydrolyzed in 2 N HCl for 10 hours at 15 pounds pressure, except for the tryptophan determination when the procedure of Greenhut *et al.* (2) was employed. Phenylalanine, leucine, isoleucine, valine, and glutamic acid were determined by techniques similar to those previously described (3), except for a slight modification of the media. Proline, arginine, methionine, threonine, serine, tyrosine, and aspartic acid were determined by the method of Henderson and Snell (4). In all determinations, the final volume was 2 ml. per tube. The organisms used for each assay were as follows: *Lactobacillus arabinosus* for valine, leucine, isoleucine, phenylalanine, tryptophan, and glutamic acid; *Leuconostoc mesenteroides* P-60, for proline, histidine, tyrosine, and aspartic acid; *Streptococcus faecalis* for methionine, threonine, and arginine; *Lactobacillus delbrueckii* 3 for lysine.

### *Results*

The water-soluble globulin was separated from the colostrum according to the procedure outlined by Smith (1), except that two additional precipitations of the protein with ammonium sulfate were employed. Sedi-

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mentation analyses were performed in a Svedberg oil turbine ultracentrifuge with a rotor speed of 50,400 R.P.M. The boundary was photographed with the aid of a cylindrical lens schlieren method in conjunction with a diagonal knife edge. With 0.15 M sodium chloride at pH 7.0 and a protein concentration of 1 and 2 per cent, the protein sedimented with a single boundary in the ultracentrifuge (Fig. 1) with a value of  $S_{20} = 7$  Svedberg units. Smith (5) reported sedimentation experiments on a colostrum pseudoglobulin which showed the presence of about 12 per cent of material with sedimentation properties different from those of the principal component.

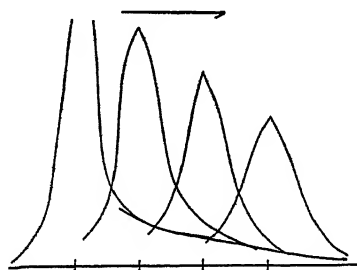


FIG. 1. Sedimentation analysis for the colostrum pseudoglobulin. Photographs were taken at 30 minute intervals and are plotted with reference to the initial base line.



FIG. 2. Electrophoresis analysis for colostrum pseudoglobulin. The analysis was made in a barbiturate-citrate buffer at pH 8.6. The descending boundary was photographed at 120 minutes.

Electrophoretic analyses were carried out in a barbiturate-citrate buffer at pH 8.6 with an ionic strength of 0.088. The protein moved as a single boundary (Fig. 2). The fact that the boundary spread considerably faster than would be expected from diffusion alone indicates some variation in mobility among the molecules.<sup>1</sup> The mobility was calculated in reference to the salt boundary and was found to be  $-2.5 \times 10^{-5}$  sq. cm. per volt per second, in agreement with the value reported by Smith (1).

The amino acid values given in Table I represent averages of many

<sup>1</sup> Electrophoresis-spreading experiments at the isoelectric point (pH 5.8) by R. A. Alberty and E. Anderson indicated a standard deviation in the mobilities of  $0.66 \times 10^{-5}$  sq. cm. per volt per second (forthcoming publication).

determinations made on several hydrolysates. Smith *et al.* (6) have reported results for leucine, valine, phenylalanine, and tryptophan on a colostrum pseudoglobulin. Our results are in good agreement with their values, except in the case of tryptophan. Brand (7) has reported amino acid analyses for a purified human  $\gamma$ -globulin. Smith (6) suggested a resemblance in amino acid content of human  $\gamma$ -globulin with colostrum pseudoglobulin with respect to the four amino acids he studied. From the figures in Table I, it is apparent that there are differences in the values for isoleucine, proline, and lysine. The results for the other amino acids

TABLE I  
*Amino Acid Values of Colostrum Globulin and Purified Human  $\gamma$ -Globulin*

	Colostrum globulin	Human $\gamma$ -globulin*
Valine.....	8.7† $\pm$ 0.2	9.7
Leucine.....	8.5 $\pm$ 0.2	9.3
Isoleucine.....	4.2 $\pm$ 0.2	2.7
Proline.....	10.0 $\pm$ 0.3	8.1
Phenylalanine.....	3.9 $\pm$ 0.2	4.6
Methionine.....	1.3 $\pm$ 0.2	1.06
Tryptophan.....	3.2 $\pm$ 0.3	2.86
Arginine.....	5.6 $\pm$ 0.2	4.8
Histidine.....	2.3 $\pm$ 0.1	2.5
Lysine.....	6.1 $\pm$ 0.2	8.1
Aspartic acid.....	9.4 $\pm$ 0.2	8.8
Glutamic ".....	12.3 $\pm$ 0.4	11.8
Threonine.....	9.0 $\pm$ 0.2	8.4
Tyrosine.....	6.7 $\pm$ 0.2	6.75
Nitrogen.....	15.9	16.03
Sulfur.....	1.1	1.02

\* Quoted from Brand (7).

† Gm. per 100 gm. of anhydrous protein.

determined are, however, somewhat similar. We found that our globulin, like human  $\gamma$ -globulin, was high in threonine and tyrosine. Repeated microbiological assays with several different organisms have shown this globulin to be high also in serine. However, a definite value for serine cannot be reported at this time as there still is considerable variability in the assay.

#### DISCUSSION

The pseudoglobulin appears to be similar to the slow moving globulins which the young calf acquires from colostrum. New-born calves deficient in the slow moving  $\gamma$ -globulins have been fed orally with normal skim milk



and different amounts of the water-soluble globulin (5). Administration of colostrum or skim milk plus the globulin causes an appreciable increase in the  $\gamma$ -globulin fraction of the serum of the new-born calf within 24 hours.

All preparations of the globulin, in agreement with the observations of Smith, have shown evidence for the presence of residual carbohydrate material. Repeated precipitation of the globulin from aqueous solution by various agents has always resulted in the carbohydrate appearing in the precipitate.

#### SUMMARY

A water-soluble globulin has been isolated from bovine colostrum.

Amino acid analyses indicate that this protein is similar in some respects to human  $\gamma$ -globulin; however, isoleucine and proline values of the colostrum globulin were markedly higher while the lysine value was lower than the corresponding values reported for human  $\gamma$ -globulin.

The protein appeared homogeneous to sedimentation and migrated as a single boundary in the electrophoresis cell under the conditions studied. The sedimentation constant was found to be  $\approx 7$  Svedberg units at pH 7.0, and the mobility  $-2.5$  sq. cm. per volt per second at pH 8.6.

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# THE HISTIDINE CONTENT OF MEAT

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WITH THE TECHNICAL ASSISTANCE OF SHIRLEY DIETERICH, MARJORY BRADFORD,  
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During the investigation of the amino acid composition of meat in the authors' laboratory, it was found that samples of the same kind of meat taken from different individual animals always had very nearly the same content of arginine, lysine, tryptophan, methionine, threonine, phenylalanine, valine, leucine, and isoleucine. It was also found that the protein in a given type of tissue had practically the same content of these amino acids, whether it came from beef, pork, or lamb. This latter finding suggests that the composition of the protein tissue structure is essentially the same in the three species of animals. Such a concept is in agreement with the findings of Beach, Munks, and Robinson (1). The similarity in the amino acid composition of animal muscle tissues has also been noted by Block and Bolling (2).

In the initial phases of the present investigation, it became apparent that there was a very significant difference in the histidine content of beef and lamb muscle tissue. Likewise, there was much less uniformity in the histidine content of different samples of the same kind of muscle tissue than there had been in the case of the amino acids previously studied. Findings such as these might be expected if a substantial part of the histidine content of muscle tissue were in some form other than protein.

At least four chemical compounds related to histidine have been found in muscle tissue. Ergothioneine and histamine are present in too small quantities to be of significance in relation to this problem. Carnosine and anserine are present in larger quantities. The relationship of these two compounds to the determination of the total histidine content of meat constitutes a part of the present investigation.

A microbiological method for the determination of histidine in meat and in other food materials is presented herewith; *Streptococcus faecalis* R is used as the test organism.

## EXPERIMENTAL

*Preparation and Hydrolysis of Meat Samples*—The dehydrated and defatted samples previously prepared for studies on the methionine content

of meat (3) were used. The ratio of the dried solids to the fresh meat was known from nitrogen determinations on the fresh and dehydrated materials. In all cases where the extracted fat contained as much as 1 per cent of the total nitrogen, this was taken into consideration in calculating the equivalence values.

Except where otherwise stated, hydrolysis of the dehydrated samples was effected by refluxing 0.5 gm. of material with 100 ml. of 6 N hydrochloric acid for 24 hours. Most of the hydrochloric acid was distilled off at reduced pressure and the solution neutralized with sodium hydroxide. Histidine determinations were also carried out on hydrolysates prepared in the same way directly from fresh meat, 2.5 to 3.0 gm. samples of finely ground meat being taken. In all cases, obvious fat and gristle were removed.

When the two procedures were applied to several samples of meat, the difference in the histidine values was well within the experimental error of the microbiological assay method.

*Determination of Histidine with Streptococcus faecalis R*—The medium for the determination of histidine with *S. faecalis R* is given in Table I. The composition of the salt solutions is as follows: Salts 1, 25 gm. of  $K_2HPO_4$ , 25 gm. of  $KH_2PO_4$ , 250 ml. of water; Salts 2, 10 gm. of  $MgSO_4 \cdot 7H_2O$ , 0.5 gm. of NaCl, 0.5 gm. of  $MnSO_4 \cdot 4H_2O$ , 250 ml. of water; Salts 3, 0.5 gm. of  $FeSO_4 \cdot 7H_2O$ , 250 ml. of water. The composition of the medium as indicated was adopted after critical comparison of variously modified media. Consistently good results were obtained when this medium was used for both histidine and threonine determinations with *S. faecalis R*. (For the determination of threonine, the threonine in the medium is replaced by an equal weight of histidine.)

The use of sodium succinate as a buffer in place of sodium acetate results in greater acid production by this organism, probably because succinate buffers the medium in a range nearer to neutrality. It has been shown by Guirard, Snell, and Williams (4) that acetate is an important nutrient for a number of lactic acid bacteria; for this reason, acetate should always be included in the medium.

Stock cultures of the organism are carried on the tryptone, peptone, tomato juice, and agar previously described (5). Solid stabs are made at monthly intervals. Weekly transfers are made from the solid medium to a liquid medium of the same composition, and serial transfers are made daily throughout the week on the liquid medium for the preparation of inocula. Cultures for inocula are incubated 18 hours and then refrigerated a few hours until used. For the preparation of inocula, the bacteria are separated from the liquid culture by centrifugation, washed twice with sterile saline, and finally diluted with sterile saline until turbidity is just

perceptible. 1 drop of the very dilute suspension is used to inoculate each assay tube.

The assay is carried out as follows: Graded amounts of pure L-histidine are added to a series of 18 mm. tubes which are to be used as standards. The range of the standards is from 0 to 50  $\gamma$  of histidine at 5  $\gamma$  intervals. Assay tubes are similarly prepared by the addition of graded amounts of neutral hydrolysates. Each assay is carried out at five different test

TABLE I  
*Medium for Determination of Histidine with Streptococcus faecalis R\**

	gm.		mg.
Glucose	40	DL-Alanine	400
Succinic acid	20	L-Arginine	400
Sodium acetate (anhydrous)	6	DL-Aspartic acid	800
	mg.	L-Cystine	400
Adenine sulfate	10	L-Glutamic acid	400
Guanine	10	Glycine	400
Uracil	10	DL-Isoleucine	400
Xanthine	10	DL-Leucine	400
Riboflavin	1	L-Lysine	400
Niacin	2	DL-Methionine	400
Pyridoxamine	0.8	DL-Phenylalanine	400
Thiamine chloride	0.4	L-Proline	400
Calcium pantothenate	0.4	DL-Serine	400
	$\gamma$	DL-Threonine	400
Biotin	2	DL-Tryptophan	400
p-Aminobenzoic acid	2	DL-Tyrosine	400
Folic acid (synthetic)	10	DL-Valine	400
	ml.		
Salts 1	10		
" 2	10		
" 3	10		

Add 12 gm. of NaOH pellets and finish neutralizing with NaOH solution, dilute to 1 liter

\* Medium for 200 cultures of 10 ml. final volume (5 ml. of above medium per culture).

levels. The standard tubes as well as the assay tubes are all prepared in duplicate. 5 ml. of the medium are added to each tube followed by water to make a total volume of 10 ml. The contents of the tubes are then mixed by shaking. Unless this is done before the tubes are autoclaved, irregularities in the results are likely to occur. The tubes are covered with aluminum caps, sterilized at 15 pounds pressure for 10 minutes, cooled, inoculated, and incubated in a constant temperature water bath at 35° for 72 hours. After incubation, the bacteria are centrifuged and 5 ml. aliquots

are titrated with 0.1 N sodium hydroxide. A typical standard curve is shown in Fig. 1.

*Determination of Histidine by Other Methods*—The medium used for the determination of histidine with *Leuconostoc mesenteroides* P-60 was the same as that described by Dunn *et al.* (6), except that norvaline, norleucine, and hydroxyproline were omitted. The general procedure for conducting the tests and for handling the bacteria was the same as that described in the present paper for the determination of histidine with *Streptococcus faecalis* R, with the exception that the incubation period was 4 days.

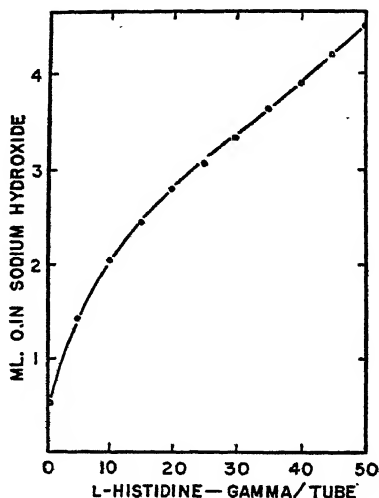


FIG. 1. Standard curve for the determination of histidine with *Streptococcus faecalis* R. Titration values are for 5 ml. aliquots from 10 ml. cultures.

Histidine was determined by the use of *Lactobacillus fermenti* 36, according to the procedure of Dunn, Shankman, and Camien (7).

Histidine was determined chemically (Pauly reaction) as described by Macpherson (8), save that the sulfanilic acid was dissolved in 5 per cent, instead of 10 per cent, hydrochloric acid. Twice the recommended amount of the sulfanilic acid solution was used in each test and, accordingly, double the amount of sulfanilic acid was employed, although the total amount of hydrochloric acid was the same as that used by Macpherson. Until this modification of the procedure was made, the values obtained for the histidine content of the copper anserine preparations did not agree at different test levels.

*Fractionation of Meat Samples*—Fresh meat was finely ground by the use of the Latapie grinder. Samples were taken for nitrogen determination

and for direct hydrolysis. A weighed portion (approximately 100 gm.) was stirred mechanically with 2 volumes of water and the protein was precipitated by the addition of 4 volumes of acetone. The precipitate was removed by centrifuging, dried *in vacuo* at 80°, and ground in a hammer mill. The supernatant liquid was filtered, concentrated at reduced pressure to remove acetone, adjusted to pH 4 with hydrochloric acid, and extracted with Skellysolve B. The aqueous phase was concentrated to dryness *in vacuo* and the residue was taken up in water, neutralized, and diluted to a suitable volume. Aliquots were taken for nitrogen determination, direct analysis, and hydrolysis.

*Preparation of Anserine*—Copper anserine was prepared from rabbit muscle tissue, as described by Schenck *et al.* (9).

TABLE II  
*Effect of Conditions of Hydrolysis on Liberation of Histidine from Meat*

	Time of hydrolysis	L-Histidine found in crude protein*
	hrs.	per cent
Refluxed with 6 N HCl	4	3.87
“ “ 6 “ “	8	3.87
“ “ 6 “ “	12	3.88
“ “ 6 “ “	16	3.90
“ “ 6 “ “	24	4.00
Autoclaved at 15 lbs., 3 N HCl	2	3.89
“ “ 15 “ , 3 “ “	4	4.03
“ “ 15 “ , 3 “ “	6	4.00

\* This is equivalent to calculating to 16 per cent protein.

### Results

Unless otherwise indicated, the data were obtained by the use of the histidine method described above in which *Streptococcus faecalis* R is the test organism.

The liberation of histidine from meat samples by hydrolysis with hydrochloric acid under various conditions was studied as a preliminary to this investigation. The data in Table II show that histidine is very readily liberated from meat samples and that it is not destroyed by prolonged hydrolysis. Values close to the maximum were obtained after autoclaving the samples for 2 hours with 3 N hydrochloric acid and also after refluxing them for 4 hours with 6 N hydrochloric acid.

Table III shows the results of recovery experiments in which pure histidine was added to hydrolysates of proteins and meat samples. The recoveries which ranged from 96 to 100 per cent were considered to be satisfactory.

The histidine content of several proteins is given in Table IV. The value of 3.04 per cent obtained for the histidine content of casein is in satisfactory agreement with the values obtained by other workers who have used microbiological methods. Guirard, Snell, and Williams (10) obtained a value of 3.2 per cent (moisture-free basis) by the use of both *Leuconostoc mesenteroides* and *Lactobacillus delbrueckii*. Dunn and Rockland (11) came to the conclusion that the histidine content of casein

TABLE III

*Recovery of Histidine Added to Hydrolysates\* (0.250 Mg. of L-Histidine Was Added to Each Hydrolysate)*

Hydrolysate	L-Histidine		Recovery of added histidine
	Present in hydrolysate	Total found	
	mg.	mg.	per cent
Bovine serum albumin.....	0.155	0.405	100.0
Fibrin.....	0.207	0.457	100.0
Beef tongue.....	0.099	0.339	96.0
" spleen.....	0.096	0.343	98.8
" liver.....	0.208	0.449	96.4
Lamb chop.....	0.128	0.372	97.6

\* The figures are averages of values obtained at five different test levels.

TABLE IV

*Histidine Content of Some Proteins*

Protein	Nitrogen content*	Histidine content*
	per cent	per cent
Casein (Difco isoelectric).....	15.56	3.04
Fibrin (Wilson Laboratories).....	16.07	2.32
Bovine serum albumin (Armour).....	16.15	3.38
Zein (commercial).....	15.20	1.22

\* Moisture-free, ash-free basis.

is 3.00 per cent (16 per cent nitrogen). Stokes *et al.* (12) reported a value of 2.8 per cent (oven-dried casein). On the other hand, the values obtained for the histidine content of casein by chemical methods are, in general, considerably lower (see Vickery and Winternitz (13)).

Average values for the histidine content of different kinds of meat are given in Table V. It will be noted that the histidine content of the protein in kidney, heart, liver, and tongue from beef, pork, and lamb was very nearly the same. A substantially higher histidine content was found in

those kinds of meat which consist primarily of skeletal muscle tissue. The statistically significant difference between the histidine content of beef loin and lamb chop is of particular interest. In contrast to this difference, data showing the similarity of these two kinds of meat with respect to their content of a number of other amino acids are given in Table VI. The figures in Table VI are averages of values obtained by the analysis of three or more samples which were known to have been taken

TABLE V  
*Histidine Content of Meat*

Kind of tissue	No. of samples	Average protein content	L-Histidine in fresh tissue			L-Histidine in crude protein,† average values
			Minimum	Maximum	Average*	
		per cent	per cent	per cent	per cent	per cent
Beef loin.....	10	21.62	0.74	0.91	0.81 ± 0.018	3.74
“ brisket.....	3	20.48	0.75	0.90	0.84	4.10
Pork loin.....	8	20.45	0.87	0.96	0.77 ± 0.032	3.76
Lamb chop.....	7	20.10	0.58	0.69	0.63 ± 0.013	3.14
Beef liver.....	4	18.88	0.49	0.52	0.50	2.64
Pork “.....	3	19.48	0.49	0.58	0.54	2.66
Lamb “.....	3	21.31	0.55	0.60	0.58	2.72
Beef tongue.....	3	17.13	0.43	0.45	0.44	2.57
Pork “.....	3	15.91	0.42	0.46	0.44	2.76
Beef heart.....	7	17.77	0.43	0.50	0.46 ± 0.010	2.59
Pork “.....	3	16.94	0.42	0.46	0.44	2.60
Lamb “.....	3	16.43	0.42	0.43	0.43	2.61
Beef kidney.....	6	17.52	0.41	0.50	0.45 ± 0.016	2.56
Pork “.....	3	15.53	0.39	0.41	0.40	2.58
Lamb “.....	3	15.68	0.40	0.41	0.41	2.61
Beef brain.....	3	10.58	0.27	0.29	0.28	2.65
“ thymus.....	3	15.93	0.27	0.29	0.28	1.76
“ spleen.....	2	18.45	0.45	0.49	0.47	2.55

\* Average values ± standard error.

† This is equivalent to calculating to 16 per cent nitrogen.

from different animals. For details of the methionine studies see Lyman *et al.* (3). Details of the determination of the other amino acids will be given in a later publication.

In order to obtain information with respect to the amount of histidine contained in animal tissues which is not combined in the form of protein, samples of finely ground fresh meat were suspended in water and the protein was precipitated with acetone. The precipitated protein and the acetone-soluble material were analyzed for histidine, the latter both before and after hydrolysis.



Fractionation of skeletal muscle tissues in this manner gave acetone-soluble fractions which contained relatively large amounts of histidine. In the case of Pork Loin 1, this amounted to a little more than one-third of the total histidine content of the original sample. As compared to beef and pork, rat muscle tissue gave an acetone-soluble fraction which contained much less histidine. The corresponding values for samples of lamb chop were intermediate. In Table VII, the histidine values for the acetone-soluble fractions are those obtained after hydrolysis.

Carnosine and anserine are typical components of skeletal muscle tissue, only small amounts of these compounds having been found in the other organs. Skeletal muscle tissue from different species varies in the relative proportion of the two compounds which they contain. Carnosine, which

TABLE VI  
*Amino Acid Composition of Muscle Tissue Expressed As Per Cent of  
Crude Protein*

Amino acid	Beef loin	Lamb chop
L-Valine.....	5.29	5.40
L-Isoleucine.....	5.84	5.74
L-Threonine.....	4.50	4.75
L-Phenylalanine.....	4.23	4.33
L-Arginine.....	6.22	6.19
L-Lysine.....	9.07	8.75
L-Methionine.....	2.47	2.41
L-Tryptophan.....	1.25	1.23
L-Histidine.....	3.74	3.14

yields natural L-histidine on hydrolysis, predominates in beef and pork, while rat muscle tissue contains relatively large amounts of anserine but very little carnosine (14-17). On hydrolysis, anserine yields methylhistidine; it is therefore important to know whether methylhistidine behaves like histidine with the microorganisms generally used for the assay of this amino acid.

Anserine was accordingly prepared from rabbit muscle tissue. After three recrystallizations of the copper derivative, the product possessed a satisfactory nitrogen and copper content.

$C_{13}H_{18}N_4O_5CuO$ . Calculated, N 17.5, Cu 19.8; found, N 17.1, Cu 19.9

Copper was removed with hydrogen sulfide and the sample was hydrolyzed with hydrochloric acid and analyzed for histidine by several different methods. Because of their similar chemical nature, anserine preparations are likely to contain small amounts of carnosine (14). The amount of

L-histidine present in the hydrolysate from this source was determined by the Pauly reaction (18), since methylhistidine does not produce a color with diazotized sulfanilic acid (14, 19).

TABLE VII  
*Histidine Distribution in Meat*

Meat sample	L-Histidine from 100 gm. fresh meat				L-Histidine in crude protein*		
	Original sample	Fraction precipitated by acetone	Fraction soluble in 60% acetone	Total accounted for	Original sample	Precipitated by acetone	Soluble in 60% acetone
	mg.	mg.	mg.	per cent	per cent	per cent	per cent
Beef Loin 1	781	567	194.9	97.6	3.74	2.97	10.60
" " 2	796	598	192.7	99.3	3.68	3.00	11.71
" " 3	760	549	176.7	95.5	3.65	2.75	8.73
Pork " 1	960	620	328.8	98.8	4.36	3.07	18.70
" " 2	684	519	170.0	100.7	3.43	3.86	9.66
" " 3	754	563	166.2	96.7	3.56	2.89	9.18
Lamb Chop 1	690	516	139.3	95.0	3.24	2.66	7.53
" " 2	632	505	96.9	95.2	3.11	2.73	5.13
Rat muscle	503	461	36.3	98.9	2.30	2.28	2.16
Beef liver	502	481	9.8	97.8	2.47	2.49	0.98
" kidney	398	404	11.0	104.3	2.37	2.60	0.84
" heart	447	443	7.5	100.8	2.44	2.60	0.60
" brain	264	254	7.0	98.9	2.54	2.63	0.93

\* This is equivalent to calculating to 16 per cent nitrogen.

TABLE VIII  
*Histidine Content of Hydrolyzed Anserine Preparation As Determined by Different Methods*

Analytical method	Histidine found*
	per cent
Chemical (Pauly reaction)	1.62
Microbiological	
<i>Leuconostoc mesenteroides</i> P-60 used as test organism	1.66, 1.63
<i>Streptococcus faecalis</i> R	2.10, 2.15
<i>Lactobacillus fermenti</i> 36	1.67

\* Values expressed as per cent of histidine in copper anserine preparation. Where two values are given, separate hydrolysates were prepared and analyzed.

The data in Table VIII, show that methylhistidine has no histidine activity at all for *Leuconostoc mesenteroides* P-60 nor for *Lactobacillus fermenti* 36, although it is slightly active for *Streptococcus faecalis* R. The

activity of methylhistidine for this latter organism is, however, too small to cause a significant error in the determination of histidine in meat.

One of the most useful methods of evaluating the reliability of microbiological assays is to make determinations by the use of more than one organism. Table IX shows the results of analyzing various kinds of meat and fractions obtained therefrom by the use of *Streptococcus faecalis* R and *Leuconostoc mesenteroides*. In only one case is there marked disagreement between the values obtained by the two methods. The higher value

TABLE IX  
Comparison of Values for Histidine Content of Meat and Meat Fractions  
Obtained by Use of Different Test Organisms

Material analyzed	L-Histidine content of 100 gm. fresh meat or fraction derived therefrom	
	Test organism	
	<i>Streptococcus faecalis</i> R	<i>Leuconostoc mesenteroides</i>
	mg.	mg.
Beef loin	781	755
Protein from beef loin precipitated by acetone	567	576
Fraction from beef loin soluble in 60% acetone, before hydrolysis	19.7	2.8
Fraction from beef loin soluble in 60% acetone, after hydrolysis	195	195
Beef liver	502	511
Protein from beef liver precipitated by acetone	481	500
Fraction from beef liver soluble in 60% acetone, before hydrolysis	6.8	6.8
Fraction from beef liver soluble in 60% acetone, after hydrolysis	9.8	8.7
Beef brain	281	275
Lamb heart	423	432

obtained for the histidine content of the unhydrolyzed acetone-soluble fraction from beef loin by the use of *Streptococcus faecalis* R suggests that this organism can make partial use of histidine in the form of carnosine. It will be noted that, after hydrolysis, the histidine values obtained by the two methods were identical. The 10-fold and greater increase in the value for the histidine content of the acetone-soluble fraction after hydrolysis indicates that most of the histidine in the acetone-soluble fraction was present in a combined form. Very little free histidine was present before hydrolysis.

In order to determine whether the acetone-soluble fraction from beef

muscle tissue contained considerable amounts of other amino acids in a combined form, the analyses indicated in Table X were carried out. Although Table X does not include data on all of the amino acids, it appears that histidine occupies a somewhat unique position. The increase in the amount of free glycine and glutamic acid after hydrolysis was probably due to the presence of glutathione. The amino acid analyses were all carried out by microbiological procedures.

TABLE X  
*Amino Acid Content of Acetone-Soluble Fraction from Beef Loin*

Amino acid determined	Amount of amino acid found	
	In non-hydrolyzed solution*	After hydrolysis*
	mg.	mg.
L-Arginine.....	15.5	15.2
L-Aspartic acid.....	1.2	5.7
L-Glutamic ".....	16.2	45.4
Glycine.....	12.7	42.6
L-Histidine.....	14.2	176.7
L-Isoleucine.....	7.9	9.5
L-Leucine.....	10.3	11.6
L-Lysine.....	3.2	6.7
L-Methionine.....	4.4	5.5
L-Phenylalanine.....	9.7	6.1
L-Proline.....	4.1	6.6
L-Threonine.....	15.4	20.1
L-Valine.....	11.3	16.3

\* Fraction obtained from 100 gm. of meat.

#### DISCUSSION

Since the hydrolysis of carnosine yields L-histidine, this fraction of the histidine in muscle tissue will be determined by any histidine method, provided that the determinations are carried out on the meat samples as a whole and not on the protein material after separation of non-protein nitrogen and other substances.

Most of the earlier investigations were designed to study the histidine content of tissue proteins and not the total histidine content of the samples. Usually the materials were treated in such a way that part or all of the carnosine content must have been lost. For example, Beach, Munks, and Robinson (1) extracted their meat samples with hot water. Osborne and Jones (20) extracted theirs first with large volumes of water saturated with toluene, then with 95 per cent alcohol, and finally with absolute alcohol. Rees (21) extracted his samples by boiling them with alcohol,

dilute acid, and finally with water. Block and Bolling (22), in one of their investigations, extracted the tissues with acetone, hot alcohol, hot benzene, and ether. It is apparent that the purpose for which the investigation was designed must be kept in mind in evaluating studies on histidine in material from animal sources.

Kuen (17) analyzed two samples of beef muscle for carnosine by several different methods and obtained values ranging from 0.380 to 0.354 per cent, depending on the method used. In terms of mg. of histidine per 100 gm. of fresh tissue, these values become 211 and 252 respectively. It thus appears that beef muscle contains sufficient carnosine to account for all of the combined histidine found in the present investigation in the acetone-soluble fractions from beef loin.

It has been shown that methylhistidine (derived from anserine) does not give rise to a significant error in the determination of histidine by any of three microbiological methods. The fact that skeletal muscle tissue from different species differs in the relative proportions of carnosine and anserine present is the probable explanation for the statistically significant difference between the average values obtained for the histidine content of beef loin and lamb chop.

#### SUMMARY

A microbiological method for the determination of histidine is described. The method was applied to the determination of histidine in meat. The meat samples studied included beef loin, round, brisket, liver, heart, kidney, tongue, brain, thymus, and spleen, as well as pork loin, liver, heart, kidney, and tongue, and lamb chops, liver, kidney, and heart.

The histidine content of the skeletal muscle tissues was higher than that in the organs such as kidney and liver. A substantial part of the histidine of the muscle tissues was found to be in a combined form which was not a part of the tissue protein. The carnosine content of muscle appears to account for this fraction of the histidine.

With the exception of skeletal muscle tissue, there was no substantial difference in the histidine content of the same kind of tissue from beef, pork, and lamb. Furthermore, the organs such as kidney, liver, heart, and tongue all had about the same histidine content.

It was found that the presence of methylhistidine does not give rise to a significant error in the determination of histidine in meat by any of three different microbiological procedures.

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## THE METABOLISM OF L-AND D-HISTIDINE BY SLICES OF LIVER AND KIDNEY\*

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Whether oxidative deamination is the favored initial step in the catabolism of histidine, as may be the case with many of the amino acids, is open to question. The supporting evidence of Krebs (1) is less striking with L-histidine than with most other natural amino acids. Kidney slices yielded somewhat more ammonia in the presence of histidine and oxygen than in the absence of either, and liver slices produced small amounts of urea and ammonia, apparently at the expense of amino nitrogen and with a measurable oxygen uptake.

According to Edlbacher and his associates (2) L-histidine is also attacked in the liver by histidase, an enzyme which leaves the  $\alpha$ -amino group intact, but hydrolyzes the imidazole ring in such a way as to permit ultimate conversion of the histidine to glutamic acid. Like glutamic acid, L-histidine is glycogenic (3, 4).

Production of urocanic acid from histidine by reductive  $\alpha$ -deamination has long been considered a possibility. Recently Edlbacher and Heitz (5) have isolated a hepatic enzyme (urocanicase) able to convert urocanic acid quantitatively to L-glutamic acid, presumably via isoglutamine, the  $\alpha$ -carbon in this instance having its origin in the imidazole ring.

That some oxidative or hydrolytic  $\alpha$ -deamination of histidine may occur seems likely. In the rat, imidazolelactic acid (6, 7) and imidazolepyruvic acid (6) can serve as dietary substitutes for histidine, and D-histidine can be inverted to form the L isomer (8). Schoenheimer, Rittenberg, and Keston (9) noted that histidine isolated from the tissues of rats fed  $N^{15}$  as ammonia contained an excess of this isotope in its  $\alpha$ -amino group.

Without doubt D-histidine is less readily utilized than the natural isomer (4, 8). It is said not to be attacked by histidase (2). According to Krebs (1) it is deaminized in the liver and kidney by D-amino acid oxidase. This has not been substantiated by tests with the reconstituted enzyme system. In such tests, Klein and Handler (10) observed only slow oxidation, Karrer and Frank (11) none.

\* The experimental data presented in this communication are taken from a dissertation submitted by Robert M. Featherstone in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Graduate College of the State University of Iowa.



Most of the studies on histidase have been made with liver extracts, which probably lack L-amino acid deaminase activity (1). The present communication records tests which were undertaken in an effort to determine whether oxidative deamination or histidase activity predominates in liver tissue and whether appreciable deamination of histidine occurs in the kidney. Slices of rat liver and rat kidney were employed. Oxygen uptake and changes in amino nitrogen and in ammonia (or ammonia and urea) were determined simultaneously. Both L- and D-histidine were used in media buffered with phosphate or with bicarbonate at the physiological pH of 7.4, as well as at pH 8.4 which Krebs found more nearly optimum for the activity of D-amino acid oxidase and Edlbacher more suitable for the activity of histidase.

#### EXPERIMENTAL

The L-histidine used in these studies was isolated as the L-histidine monohydrochloride monohydrate from spray-dried blood by the method of Cox, King, and Berg (12). It was racemized as directed by Duschinsky (13) and the D-histidine was prepared from the racemate as the monohydrochloride monohydrate, essentially as outlined by Conrad and Berg (8). Solutions of 2 gm. of the monohydrochloride monohydrate and 1 equivalent of hydrochloric acid in 100 cc. of water gave  $[\alpha]_D^{25} = +8.32^\circ$  for the L and  $-8.20^\circ$  for the D form (cf. (8, 14)). Both isomers melted at  $251-253^\circ$ . None of the preparations responded to Sullivan's naphthoquinone test for cystine, which Darby and Lewis (15) have reported may contaminate preparations of histidine monohydrochloride, particularly if the histidine is not first separated as the dihydrochloride, as was done in our procedure. The ninhydrin "carboxyl nitrogen" method of assaying free amino acids (16) yielded 99 per cent or more of the calculated "carboxyl nitrogen" on aliquots containing as little as 0.04 mg. of histidine. Since the amino acid was to be used in a system sensitive to mercury and its preparation had involved its isolation initially as the mercuric sulfate complex, it was tested for residual mercury. No evidence that even minute traces were present could be obtained by the Reinsch,  $\text{SnCl}_2$ , or dithizone tests, or by spectrographic analysis. For use in the tissue slice studies the histidine monohydrochloride monohydrate was dissolved in ammonia-free water together with an equivalent weight of sodium hydroxide. The requisite amount of histidine was employed to provide a 0.02 M solution after its admixture with the buffered medium in which the tissue was suspended.

The tissue slices were prepared from the livers or kidneys of rats from Sprague-Dawley stock which had been maintained on a diet of Purina dog chow. The animals were killed by a blow on the head. The slices were suspended in phosphate or bicarbonate media, prepared as directed by

Krebs and Henseleit (17). Addition of the histidine solution caused no appreciable change in pH.

Standard manometric procedures were used to determine oxygen utilization. Data for experiments in which bicarbonate buffers were used were obtained by employing the apparatus described by Dixon and Keilin (18). The gassing in these instances was with a mixture of 95 per cent oxygen and 5 per cent carbon dioxide, rather than with the usual 100 per cent oxygen. Oxygen data were recorded for a period of  $1\frac{1}{2}$  or 3 hours before the slices were treated with acid from the side sac. The slices were then removed for weighing, and the solutions for analysis.

When urea and ammonia were determined together, as was usual in the studies with liver slices, the aliquots were incubated for 30 minutes with urease in the form of a finely ground Arlco tablet. The total ammonia was measured by distillation and titration, essentially as described by Keys (19). The same procedure was used for estimating ammonia in the tests with kidney slices. The standard acid and alkali solutions were checked against each other daily and the normality of the standard alkali was redetermined each month by titration against potassium acid phthalate. Blank determinations were made routinely on all other solutions and reagents used in the study and corrections were applied as found necessary.

Amino nitrogen was measured by the ninhydrin "carboxyl nitrogen" method (16). To verify the calculated quantity of amino nitrogen added to the respirometers, the same volumes of amino acid, buffer solution, and acid as were used in the studies were mixed together and analyzed.

#### RESULTS AND DISCUSSION

The data, summarized in Tables I and II, were calculated conventionally as  $Q$  values,  $Q$  representing the apparent change per mg. of dry tissue per hour in c.mm., of oxygen, or non-gaseous substance expressed in c.mm., 1 millimole being considered equivalent to 22,400 c.mm. The average  $Q$  (experimental minus control) values tabulated represent differences between averages of triplicate or quadruplicate tests in which the amino acid was added to the flasks, and averages of simultaneous tests in which slices were incubated with the buffered medium alone. A plus sign indicates that the average oxygen uptake by the flasks containing the amino acid was the greater. To avoid confusion, readings of the Dixon-Keilin differential manometer were expressed in the same way, even though this is contrary to the usual practice.

$Q_{\text{urea}} + \text{NH}_3$  includes both urea and ammonia expressed as ammonia,  $Q_{\text{NH}_3}$  only ammonia. A plus difference indicates a greater average content, after incubation, in the experimental than in the control flasks, a negative difference the converse.

Some free amino acid nitrogen (carboxyl nitrogen) was always found in

the media of the control flasks after incubation. In determining changes in the media of the experimental flasks, obviously the added amino acid nitrogen was considered. If it is assumed that the liberation of amino acid nitrogen from tissue is not appreciably altered by the presence of added amino acid, then a minus difference in  $Q_{\text{amino N}}$  values may be said to indicate utilization of amino acid nitrogen; a plus difference would indicate either lack of utilization or its masking by an excessive simultaneous liberation of amino acid nitrogen from the tissue.

TABLE I

*Utilization of Oxygen and Amino Nitrogen and Production of Ammonia and Urea by Slices of Rat Liver Incubated with Media Containing L- or D-Histidine*

Series No.	Average Q (experimental minus control)			pH	Buffer	Special addition to media
	O <sub>2</sub>	Urea + NH <sub>3</sub>	Amino N			
L-Histidine						
I	-0.38	+11.10	-6.26	7.4	PO <sub>4</sub>	
III	-6.51	+3.20	-10.28	8.4	"	
V	0.00	+5.97	-5.05	7.4	HCO <sub>3</sub>	
VII		+6.24	-6.72	8.4	"	
VIII		+17.07	-5.57	7.4	"	Octyl alcohol
IX	+0.60	+13.85	-6.91	8.4	"	" "
X		+8.90	-10.30	8.4	"	" "
XI		+15.50	-9.54	8.4	"	" "
D-Histidine						
II	-4.81	-2.28	-2.35	7.4	PO <sub>4</sub>	
IV	+2.48	+0.37	-2.12	8.4	"	
VI	-1.73	-3.32	-2.81	7.4	HCO <sub>3</sub>	

Table I records the results of several series of liver slice tests with L- and D-histidine. In every trial with L-histidine an increase in urea plus ammonia occurred. In only one of the four series in which oxygen uptake was measured was there any indication that oxygen was consumed. The exception (Series IX) was one in which the medium was saturated with octyl alcohol which, according to Krebs (1), inhibits the activity of L-amino acid deaminase. In none of the tests in which the L-histidine media were saturated with octyl alcohol was there evidence of suppressed ammonia and urea production. Comparisons with control tests indicated quite the contrary. Disappearance of amino nitrogen was also greater. The data suggest that the changes were chiefly non-oxidative.

The decrease in amino nitrogen indicates that the change may not be

confined to the production of glutamic acid by histidase, which liberates 2 equivalents of ammonia but does not affect the amino nitrogen. Non-oxidative metabolism of the glutamic acid produced, formation of urocanic acid, conversion to histamine, and production of peptides are all possibilities, but the data afford no indication as to which or how many of these may be involved.

Trial tests on DL-alanine without octyl alcohol strongly suggested that oxidative deamination occurred. In the presence of octyl alcohol considerable inhibition of ammonia and urea formation and of amino nitrogen utilization was observed.

TABLE II  
*Utilization of Oxygen and Amino Nitrogen and Production of Ammonia by Slices of Rat Kidney Incubated with Media Containing L- or D-Histidine*

Series No.	Average Q (experimental minus control)			pH	Buffer	Special addition to media
	O <sub>2</sub>	NH <sub>3</sub>	Amino N			
D-Histidine						
I	-2.70	+2.74	+1.52	7.4	PO <sub>4</sub>	As <sub>2</sub> O <sub>3</sub>
III	-1.30	+1.12	-1.84	8.4	"	
V	+2.94	+3.03	-6.95	7.4	HCO <sub>3</sub>	
VII	+0.28	+0.05	-17.10	7.4	"	
L-Histidine						
II	-3.17	+0.23	+1.49	7.4	PO <sub>4</sub>	
IV	-4.30	-0.75	+2.17	8.4	"	
VI	0.00	-0.26	-6.34	7.4	HCO <sub>3</sub>	

The data on D-histidine show little or no production of urea plus ammonia and a loss of amino nitrogen much smaller than with L-histidine. The evidence fails to support either oxidation by D-amino acid oxidase or cleavage by histidase. The former observation agrees with the evidence which Klein and Handler (10) and Karrer and Frank (11) obtained in tests with reconstituted D-amino acid oxidase, the latter observation with the claim of Edlbacher (2) that histidase does not attack D-histidine.

Table II records the data obtained in experiments with kidney slices and D- or L-histidine. When D-histidine was tested in the phosphate-buffered media, no oxygen uptake occurred and little or no amino nitrogen disappeared; in the bicarbonate media, the oxygen uptake, ammonia production, and diminution of amino nitrogen all favored the possibility that some oxidative deamination may have taken place. When arsenious

oxide was added to the system, the disappearance of amino nitrogen became marked, but oxygen uptake and ammonia formation diminished sharply. According to Krebs arsenious oxide does not interfere with deamination by D-amino acid oxidase, but prevents oxidation of the deaminized residue beyond the ketonic acid stage.

Comparative tests indicated that DL-alanine (in phosphate-buffered media) was much more vigorously attacked. A greater loss of amino nitrogen was observed when arsenious oxide was added to the system, but the oxygen uptake and the production of ammonia were not as markedly affected as in comparable tests with D-histidine.

Incubation of L-histidine with kidney slices induced no oxygen uptake and no appreciable production of ammonia. These results are contrary to those noted in a single test by Krebs in which he obtained differences in  $Q$  values of +2.10 for oxygen, +0.91 for ammonia, and -2.40 for amino nitrogen. Krebs' contention that D-histidine is more readily deaminated in the kidney than is the L isomer is apparently based solely on differences in  $Q_{NH_4}$  of +8.42 for D-histidine and +1.85 for the L-amino acid (1).

#### SUMMARY

Data obtained on oxygen uptake, amino nitrogen diminution, and urea and ammonia production, upon incubating media containing histidine with liver slices, seem to indicate that histidase plays a more important rôle than L-amino acid deaminase in the metabolism of the natural isomer of this amino acid by this tissue. Comparable tests indicate that the D isomer of histidine is not attacked by histidase and is not readily oxidized by D-amino acid oxidase.

Analogous tests with kidney slices afford some indication that D-histidine may undergo slight oxidative deamination in the kidney, but no evidence that L-histidine is either oxidatively deaminized or split hydrolytically in that organ.

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# METHIONINE SYNTHESIS IN NEUROSPORA. THE ISOLATION OF CYSTATHIONINE\*

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Among artificially produced biochemical mutants of *Neurospora*, those which have lost the ability to synthesize methionine form the largest class. At the present writing 87 occurrences of the *methionineless* character have been observed in this laboratory following treatment of wild type spores with high frequency radiations (1) or mustard gas (2). *Methionineless* mutants differ from wild type *Neurospora* in that they fail to grow on a medium containing only sugar, inorganic salts, and biotin, but do grow if, in addition to these constituents, methionine is supplied. In many of the mutants failure of methionine synthesis results from a block in the reduction of sulfate, which, except for a trace of biotin, is the sole source of sulfur in the basal medium. These strains can utilize reduced forms of inorganic sulfur for growth, as well as methionine and other organic sulfur compounds. On the other hand, some of the mutants require organically bound sulfur for growth, an indication that in these strains the block in methionine synthesis comes at a later stage than sulfate reduction. Similar classes of methionine-requiring mutants have been reported in the mold *Ophiostoma* by Fries (3) and in *Escherichia coli* by Lampen *et al.* (4-6).

The present study is concerned with four strains which are unable to carry out certain of the terminal steps in methionine synthesis. Evidence is presented showing that the synthesis proceeds through a series of gene-controlled reactions involving cysteine, cystathionine, and homocysteine as intermediates. Of particular interest is the fact that, as a result of genetic blocking, a precursor of methionine accumulates in cultures of one of the strains. The precursor has been isolated and shown to be chemically and biologically indistinguishable from synthetic L-cystathionine.

The results of this investigation were presented at the meeting of the American Society of Biological Chemists in Chicago, May 21, 1947 (7).

\*The subject matter of this paper has been undertaken in cooperation with the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces. The opinions or conclusions contained in this report are those of the author. They are not to be construed as necessarily reflecting the views or indorsement of the War Department.

This investigation was also supported in part by the Nutrition Foundation, Inc.



*Materials and Methods*

*Media*—The basal medium used in the experiments has the following composition, in gm. per liter: ammonium tartrate 5, ammonium nitrate 1, monobasic potassium phosphate 1, magnesium sulfate ( $7H_2O$ ) 0.5, sodium chloride 0.1, calcium chloride 0.1, sucrose 20, biotin  $5 \times 10^{-6}$ , and the following trace elements (added as salts), in mg. per liter, B 0.01, Mo 0.02, Fe 0.2, Cu 0.1, Mn 0.02, Zn 2.0.

Stock cultures of the mutants were carried on agar slants of the following composition, in gm. per liter: potassium tartrate 5, sodium nitrate 4, monobasic potassium phosphate 1, magnesium sulfate ( $7H_2O$ ) 0.5, sodium chloride 0.1, calcium chloride 0.1, glycerol 20, hydrolyzed casein 0.25, Difco yeast extract 5, Difco malt extract 5, DL-methionine 0.025, agar 15.

Growth was measured by inoculating the mold into 20 ml. of medium in 125 ml. Erlenmeyer flasks, incubating for 72 hours at  $25^\circ$ , drying the mycelium at  $90^\circ$ , and weighing to the nearest 0.5 mg.

*Mutant Strains*—The isolation numbers of the mutants reported in this paper are 38706, H98, 36104, and 39816. For convenience these will be referred to as *methionineless-1*, *methionineless-2*, *methionineless-3*, and *methionineless-4*, respectively, abbreviated *me-1*, *me-2*, etc. The same numbers can be used without confusion to designate the distinguishing gene of each strain. Thus, for example, strain *me-1* carries a mutation of gene *me-1*, but has all other known genes in their normal forms.

Strains *me-1*, *me-3*, and *me-4* were obtained from wild type *Neurospora crassa* after irradiation with ultraviolet light, while strain *me-2* was isolated (by Dr. Frank Hungate) from material exposed to x-rays. A genetic investigation of strains *me-2* and *me-3* has been carried out by Buss (8), who reports that both mutants differ from the wild type by different single genes. *Me-1* and *me-4* have been studied by Dr. B. Phinney in this laboratory, who finds that they are single gene mutants differing from strains *me-2* and *me-3*. Crosses between strains *me-1* and *me-4* are infertile (Phinney, personal communication), and the heterocaryon test for allelism (9) has so far given negative results; so that at present there is no genetic evidence that these two strains differ. In view of the differences in nutritional requirements to be reported below, however, there is little doubt that these strains do differ genetically, and in what follows they will be considered so to differ.

*Compounds*—I am greatly indebted to Dr. Vincent du Vigneaud for samples of synthetic L-cystathionine, D-cystathionine, L-allo-cystathionine, and D-allo-cystathionine. I wish also to thank Dr. H. Borsook and Dr. J. Dubnoff for samples of L-homocysteine and L-homocystine.

DL-Homocysteine thiolactone hydrochloride was prepared from DL-homo-

cystine by the method of Riegel and du Vigneaud (10). The methionine, cystine, and cysteine hydrochloride were commercial preparations.

#### EXPERIMENTAL

*Growth of Mutants on Homocysteine and Cysteine*—It is known from the work of du Vigneaud *et al.* (11) that the rat can methylate homocysteine to form methionine. If a similar reaction takes place in *Neurospora*, and if the reaction is gene-controlled, then one might expect to find two classes of *methionineless* mutants with respect to homocysteine utilization, those which can use homocysteine for growth and those which cannot. A preliminary survey of a number of *methionineless* strains showed that these classes do in fact exist. Of the four strains reported here, strain *me-1* fails to grow on the basal medium supplemented with homocysteine, while the other three mutants utilize homocysteine readily (Table I). Strain *me-1* does not

TABLE I  
Activity of Sulfur-Containing Amino Acids for Four *Neurospora* Mutants

Amino acid	Dry weight of mold after 72 hrs. at 25°			
	Strain <i>me-4</i>	Strain <i>me-3</i>	Strain <i>me-2</i>	Strain <i>me-1</i>
	mg.	mg.	mg.	mg.
None.....	0	0	0	0
DL-Methionine, 1 mg.....	22.0	54.0	66.0	57.5
L-Homocysteine, 1 mg.....	23.0	35.0	32.0	0
DL-Homocysteine thiolactone·HCl, 2 mg....	16.5	36.5	41.0	0
L-Homocystine, 2 mg.....	0	0	0	0
L-Cysteine·HCl, 1 mg.....	25.0	0	0	0
L-Cystine, 1 mg.....	27.0	0	0	0

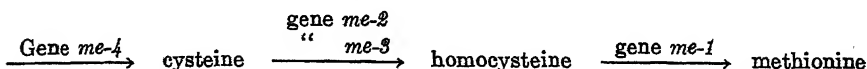
grow on homocysteine even with choline or betaine supplied additionally, suggesting that the transfer of methyl, rather than its synthesis, is blocked in this mutant.

The thiolactone of homocysteine is somewhat less active than homocysteine in promoting growth, while homocystine is inactive. Evidently the last compound is not appreciably reduced to homocysteine by the mold under the conditions of these experiments. The observed activity of homocysteine for strains *me-2* and *me-3* is approximately 0.25 that of DL-methionine, calculated on a molecular basis. This represents a minimal value, since a significant quantity of homocysteine is undoubtedly oxidized to homocystine in the course of a 3 day aerobic growth experiment.

The foregoing results indicate that the normal (wild type) form of gene *me-1* controls the methylation of homocysteine and that genes *me-2*, *me-3*, and *me-4* control earlier steps in the synthesis of methionine. The results

obtained when cystine (or cysteine) is supplied throw light on the functions of the last three genes. It is found that strain *me-4* responds to cystine and cysteine, whereas the other strains do not (Table I). It follows that the synthesis of cysteine is blocked in strain *me-4* and that cysteine can serve as a precursor of methionine. The activity of cystine (calculated as cysteine) for strain *me-4* is approximately equal to that of methionine.

The conversion of cysteine to methionine is dependent on the normal functioning of genes *me-1*, *me-2*, and *me-3* since strains carrying mutant forms of these genes fail to bring about the conversion. These conclusions are summarized in the following diagram.



Inspection of Table I shows that strain *me-4* grows at a slower rate than do the other mutants tested. A 3-fold increase in the concentration of cystine or methionine only slightly increases the growth rate over that shown in Table I. A depressed growth rate has been found in a number of other *cystineless* mutants, although this is not characteristic of all such strains. The phenomenon is being investigated.

*Conversion of Cysteine to Homocysteine*—As mentioned above, the genetic findings of Buss showed genes *me-2* and *me-3* to be non-allelic. It was of considerable interest to determine whether a difference in function could be demonstrated between them. Aqueous extracts of each mutant were therefore tested for their ability to support the growth of the other strain. It was found that extracts of strain *me-2*, or the medium in which it has grown, contain a factor which can be utilized for growth by strain *me-3* (but not by strain *me-2*). The finding suggested that a precursor of homocysteine accumulates in cultures of strain *me-2* as a result of the genetic blocking.



The situation is identical in principle with that encountered in the accumulation of anthranilic acid by strain 10575 of *Neurospora* (12), of monomethylaminoethanol by strain 47904 (13), and of an unidentified precursor of nicotinic acid by strain 4540 (14).

The isolation of the intermediate was accomplished by the following procedure.

*Isolation of Cystathionine*—Strain *me-2* was inoculated into 5 gallon Pyrex carboys containing 16 liters of basal medium plus 0.4 gm. of DL-methionine. The carboys were incubated at 25° under forced aeration for a period of 7 to 10 days. At the end of this time the mycelium was collected in a basket

centrifuge and washed with water. The moist weight of the mycelium at this stage varied from 290 gm. (7 days incubation) to 360 gm. (10 days incubation) per carboy.

Bioassay with strain *me-3* having shown that the active substance is more highly concentrated in the mold than in the medium, the latter was ordinarily discarded. In one instance the medium was worked up in parallel with the mycelium from the same carboy. The yield of pure material from the mycelium was over 10 times that obtained from the medium.

The mycelium was dispersed in water in a Waring blender and poured into 10 liters of boiling water per kilo of moist mold. After 10 minutes the suspension was filtered through cloth and the extraction repeated. The combined filtrates were brought to pH 5 with dilute HCl and placed in the cold room. The next day the precipitate was filtered off through a layer of infusorial earth and discarded. The clear filtrate was neutralized and concentrated under reduced pressure to a volume of 2.6 liters per kilo of the original mycelium. 1 volume of 95 per cent alcohol was added with stirring, and the mixture was placed in the refrigerator overnight. The precipitate was removed by filtration and discarded.

The filtrate was concentrated to 550 ml. per kilo of original mycelium and 5 volumes of alcohol were added. After standing in the cold overnight the precipitate was collected in the centrifuge and washed with successive small portions of ice-cold water until the washings were colorless. The residue was dissolved in hot water, treated with charcoal, and concentrated under reduced pressure until crystallization began. 3 volumes of alcohol were added and the mixture refrigerated overnight.

The precipitate was collected by centrifuging and was recrystallized from water. The colorless octagonal prisms were washed with cold water and alcohol and dried with ether. Yield, 360 mg. per kilo of moist mycelium. The yield from one carboy was doubled by supplementing the basal medium with 0.2 gm. of L-cystine and 0.2 gm. of DL-homoserine in addition to the usual methionine supplement. I am indebted to Dr. Marguerite Fling for the synthetic homoserine used in this experiment.

Elementary analysis of the isolated material showed the following.

$C_7H_{14}O_4N_2S$ .	Calculated.	C 37.78, H 6.35, N 12.59, S 14.42
	Found.	" 37.68, " 6.46, " 12.47, " 14.80

The material gives a strong ninhydrin test. It darkens at 270° and melts with decomposition at 301°. The specific rotation,  $[\alpha]_D^{25}$ , of a 1 per cent solution in 1 N HCl is  $+26^\circ \pm 2^\circ$ . These values are in agreement with those obtained by du Vigneaud *et al.* (15) for synthetic L-cystathionine.

55 mg. of the isolated material were treated with benzoyl chloride by the method of Anslow *et al.* (16), yielding 50 mg. of the dibenzoyl derivative.

$C_{21}H_{23}O_6N_2S$ . Calculated, N 6.51; found, N 6.36

The compound melted at 228–229°, in agreement with the reported melting point of dibenzoyl-L-cystathionine (15). This finding, taken together with the observed specific rotation of the free amino acid, defines the configuration of *Neurospora* cystathionine as of the L, or natural, configuration throughout (16).

TABLE II  
*Biological Activity of Natural and Synthetic L-Cystathionine*

Cystathionine per 20 ml. medium	Growth of strain <i>me-3</i>	
	On synthetic cystathionine	On natural cystathionine
mg.	mg.	mg.
0	0.0	0.0
0.2	10.0	10.0
0.4	20.0	20.0
0.6	28.5	33.0
0.8	38.0	40.5
1.0	44.5	44.0

TABLE III  
*Activity of Cystathionine Isomers for Neurospora Mutants*

L-Cystathionine was isolated from *Neurospora*; the other isomers were synthetic. Strain *me-4* was incubated at 25° for 96 hours, all others for 72 hours. Concentration of amino acid, 1 mg. per 20 ml.

Isomer	Dry weight of mold			
	Strain <i>me-4</i>	Strain <i>me-3</i>	Strain <i>me-2</i>	Strain <i>me-1</i>
	mg.	mg.	mg.	mg.
L-Cystathionine.....	26.0	50.5	0.0	0.0
D-Cystathionine.....	0.0	0.0	0.0	
L-Allocystathionine.....	4.0	0.5	0.0	
D-Allocystathionine.....	3.0	0.0	0.0	

**Biological Activity**—As a final confirmation of the identity of the isolated compound, its biological activity was compared with that of synthetic L-cystathionine with strain *me-3* as a test organism. The results (Table II) show that the activities of the two compounds are identical within the limits of error. The molecular activity of cystathionine for strain *me-3* is approximately 0.6 that of methionine.

Through the courtesy of Professor du Vigneaud, who supplied synthetic material, it was also possible to test the activity of the three unnatural optical isomers of cystathionine. The results are summarized in Table III.

Only L-cystathionine is active for strain *me-3*, showing that only this isomer can be cleaved by *Neurospora* to yield homocysteine. Both L- and D-allo-cystathionine are slightly active for strain *me-4*, indicating that these may serve to a limited extent as sources of cysteine for the organism.

#### DISCUSSION

S-( $\beta$ -Amino- $\beta$ -carboxyethyl)-homocysteine, later named cystathionine by Binkley and du Vigneaud (17), was first suggested by Brand *et al.* (18) as a possible intermediate in the biological conversion of methionine to cystine. Previously, the structure had been tentatively assigned by Küster and Irion (19) to a substance they isolated from wool, following prolonged treatment with sodium sulfide. Later the same structure was suggested by Horn and Jones (20) for an amino acid isolated from seleniferous grains in isomorphous combination with the selenium-containing analogue.

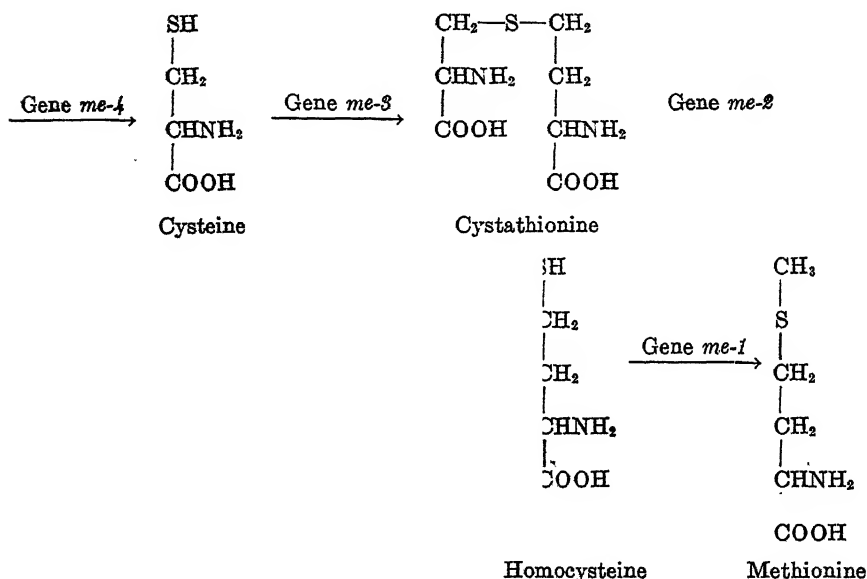
In recent years the problem has been vigorously attacked by du Vigneaud and his coworkers, who have accomplished the synthesis of optically active cystathionine as well as of active allocystathionine (15, 16). The same workers have brought forward important experimental support for the hypothesis of Brand *et al.* concerning the rôle of cystathionine in the methionine-cystine conversion (15-17, 21, 22).

The results of the present study indicate that the most probable pathway of conversion of cysteine S to methionine S in *Neurospora* is by way of the gene-controlled steps shown in Diagram 1. Mutation of the indicated genes, singly, to inactive forms would result in strains with the nutritional requirements described in this paper. This pathway is exactly the reverse, with respect to the S-containing intermediates, of that which has been proposed for the conversion of methionine S to cysteine S in the mammal. In view of the data of Lampen *et al.* (6) it is likely that a similar sequence of reactions operates in *Escherichia coli*; in this organism, however, a mutant corresponding to strain *me-3* has not yet been found.

Binkley and du Vigneaud (17) found that cysteine is formed from homocysteine and serine in the liver, presumably through the intermediate formation of cystathionine, while Stetten (23) has shown that N<sup>15</sup>-labeled serine gives rise to isotopic cystine in the rat. The question therefore arises whether serine is produced in the homocysteine-yielding step in *Neurospora*. Present evidence indicates that serine is not produced in this step by direct hydrolysis of L-cystathionine into 1 molecule of serine and 1 molecule of homocysteine. If this were the case, it would be impossible to obtain a serine-requiring mutant which is not at the same time *methionineless*, since a block in serine synthesis would necessarily involve either the cleavage of cystathionine or a step in the synthesis of cystathionine. A monogenic, *serineless* mutant of *Neurospora* has, however, been described by

Hungate (24). This mutant does not require exogenous methionine for growth, although a slight stimulatory effect of methionine, of doubtful significance, was found. It is probable, therefore, that serine and homocysteine are not products of the same reaction. Consistent with this conclusion is the result of a single experiment by the author in which the *serineless* mutant was found not to respond to L-cystathionine. The evidence does not exclude the possibility of serine production indirectly from cystathionine by phosphorolytic cleavage, yielding phosphoserine, from which serine might be subsequently liberated. Such a phosphorolysis

DIAGRAM 1



would be analogous with that which, according to the experiments of Binkley (22), is undergone by L-cystathionine in the cysteine-yielding reaction in liver preparations.

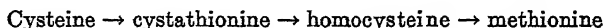
Strain *me-4*, as well as all other known *cystineless* strains of *Neurospora*, grows in the absence of added cystine if methionine is supplied. This means that in the mold, as in animals, methionine can be utilized for cystine formation. Similarly, cystathionine and homocysteine can also be converted to cystine by *Neurospora*. By analogy with the rat, it seems probable that the conversion of methionine S to cysteine S is simply the reverse of the synthesis of methionine from cysteine, shown in Diagram 1. However, no direct evidence has yet been obtained on this point. It is

conceivable that the formation of cystine from methionine in the mold proceeds over a route not involving homocysteine or cystathionine. A test of this hypothesis should be possible by the use of double mutants.

I wish to thank Dr. A. J. Haagen-Smit and Dr. G. Oppenheimer for the microanalyses reported here. Valuable assistance was rendered to the author by Dr. Marguerite Fling and Dr. B. Phinney.

#### SUMMARY

Four radiation-induced, single gene mutants of *Neurospora* which are unable to synthesize methionine from the constituents of the basal medium are described. The synthesis is blocked at a different stage in each mutant. Strain *me-4* can utilize cystine, cystathionine, and homocysteine for growth, in addition to methionine. Strain *me-3* can utilize cystathionine, homocysteine, and methionine, but not cystine. Strain *me-2* grows on homocysteine or methionine, but not on cystine or cystathionine. Strain *me-1* utilizes only methionine. A substance is produced by strain *me-2* which is active for strains *me-3* and *me-4* but not for strains *me-2* and *me-1*. The substance has been isolated and identified as L-cystathionine on the basis of the following criteria: elementary analysis, optical rotation, decomposition temperature, melting point of the dibenzoyl derivative, and biological activity. The results indicate that L-cystathionine is a normal intermediate in the synthesis of methionine by *Neurospora* and that cysteine S is converted to methionine S over the following pathway.



Each step is under genic control. Of the four optical isomers of cystathionine, only L-cystathionine is cleaved to homocysteine.

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# INTERRELATIONSHIP BETWEEN *p*-AMINOBENZOIC ACID AND PTEROYLGLUTAMIC ACID AS GROWTH FACTORS FOR LACTOBACILLI\*

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The elucidation of the structure of pteroylglutamic acid (PGA) (*Lactobacillus casei* factor, vitamin B<sub>12</sub>) has shown that *p*-aminobenzoic acid (PABA) is a component of the PGA molecule (1). Since earlier work has treated PABA and PGA as distinct growth factors for bacteria (2), it is now necessary to study the relationship in the utilization of these substances by bacteria. It has been shown that many bacteria can synthesize PGA (3-7) and in some cases this has been related to the PABA content of the medium (5, 6). Recent findings also indicate that sulfonamides inhibit the synthesis of PGA from PABA by enterococci (8).

*Lactobacillus casei* and *Lactobacillus arabinosus* have been used in the present work to study the synthesis of PGA from PABA and to show only partial replacement of PABA as a bacterial growth factor by PGA.

## EXPERIMENTAL

### Methods

For experiments with *Lactobacillus casei* (American Type Culture Collection No. 7469) and for measurement of PGA a modification of the Landy and Dicken medium (9) was used. Asparagine was omitted and the biotin content was reduced to 2  $\gamma$  per liter. The glucose and sodium acetate concentrations were increased to 40 gm. each per liter. Experiments with *Lactobacillus arabinosus* 17-5 (American Type Culture Collection No. 8014) were conducted with the nicotinic acid test medium of Krehl, Strong, and Elvehjem (10), modified by the deletion of PABA and the inclusion of 400  $\gamma$  of nicotinic acid per liter. In both of the above media, Difco vitamin-free casamino acid mixture<sup>1</sup> was used as the acid-hydrolyzed casein. For inocula the organisms were transferred from yeast agar stabs (10) to 10 ml. of the diluted medium, to which had been added the missing

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<sup>1</sup> This material was generously furnished by Dr. H. W. Schoenlein of the Difco Laboratories, Inc.

growth factor and 5 mg. each of Difco yeast extract and Wilson liver concentrate 1:20.<sup>2</sup> The bacteria were incubated for 20 to 24 hours at 37°, centrifuged, washed, and resuspended in an equal volume of saline. This was diluted 20-fold and 1 drop was used for inoculation of each tube. All tests were conducted in 18 × 150 mm. Pyrex test-tubes, with a total volume of 10 ml. per tube.

For assays the tubes were incubated at 37° for 60 to 65 hours and the acid produced was titrated with 0.1 N NaOH with brom-thymol blue as indicator. For studies on the synthesis of PGA from PABA by *Lactobacillus arabinosus* the cultures were steamed for 20 minutes after incubation, neutralized to pH 6.9, diluted for analysis, and filtered. Experiments in which cells and media were analyzed separately were carried out similarly after preliminary centrifugation and separation. The materials under study were then analyzed for PGA with *Lactobacillus casei* grown in the modified Landy-Dicken medium.

Synthetic PGA, pterioic acid, *p*-aminobenzoylglutamic acid (PABG), and thymine were employed.<sup>3</sup> The sample of PGA used in these studies contained 2.2 per cent of free arylamine (calculated as PABA), whereas only 1 per cent of free arylamine was found in the pterioic acid (11).

*Synthesis of PGA from PABA by Lactobacillus arabinosus*—The distribution of free PGA in the cells and medium of the *L. arabinosus* cultures which were incubated with known amounts of PABA was studied first. No assays were made of any bound PGA (conjugate) in any of the experiments. In order to obtain measurable amounts of PGA it was necessary to add PABA far in excess of that needed for maximal acid production. The results in Table I show that most of the free PGA formed by *L. arabinosus* was present in the culture medium. This differs from the findings with *Streptococcus lactis* R in which almost all of the PGA formed after incubation with pterioic acid was present in the cells (4).

Since excess thymine can replace PGA for *Lactobacillus casei* and support one-half maximal growth of this organism (12, 13), the effect of thymine on the synthesis of PGA from PABA by *L. arabinosus* was examined. PGA values as determined in the analyses by *L. casei* were corrected for the effect of the thymine which was carried over. Table II shows that the presence of 5 γ of thymine per tube had no significant effect on the synthesis of PGA from PABA by *L. arabinosus*. However, in other experiments in this

<sup>2</sup> This concentrate was kindly supplied by Dr. Stanley W. Hier of Wilson and Company.

<sup>3</sup> We are indebted to Dr. E. L. R. Stokstad of the Lederle Laboratories Division of the American Cyanamid Company, for the pteroylglutamic acid and related compounds, and to Dr. J. A. Aeschlimann of Hoffmann-La Roche, Inc., for the thymine used in these studies.

laboratory it was observed that thymine (5 to 10  $\gamma$  per tube) did have a slight stimulatory action on the growth of *L. arabinosus* in the presence of suboptimal amounts of PABA.

The effect of the amino acid source in the culture medium and of time of incubation on the synthesis of PGA during growth of *Lactobacillus arabinosus* with PABA is shown in Table III. Most of the synthesis of PGA occurred in the first 20 hours of incubation. This corresponds approximately to the end of the logarithmic growth period for *L. arabinosus*. This

TABLE I

*Distribution of Free Pteroylglutamic Acid in Cells and Medium after Incubation of Lactobacillus arabinosus with p-Aminobenzoic Acid*

PABA added	Free PGA content after incubation (40 hrs.)	
	Medium	Cells
m $\gamma$	m $\gamma$	m $\gamma$
25	4	*
50	8	0.6
100	24	1.1
200	64	2.8

\* This value was too small to be measured accurately.

TABLE II

*Effect of Thymine upon Synthesis of Free Pteroylglutamic Acid from p-Aminobenzoic Acid by Lactobacillus arabinosus*

PABA added	Free PGA content of cultures after incubation (40 hrs.)	
		In presence of 5 $\gamma$ thymine
m $\gamma$	m $\gamma$	m $\gamma$
50	11	11
100	28	32
200	90	95
500	85	99

is similar to the synthesis of PGA from pterioic acid by *Streptococcus lactis* R, which reached its maximum in about 18 hours (4). In other experiments, *L. arabinosus* was incubated for shorter periods of time with similar amounts of PABA in Medium A. It was found that in 7 to 8 hours, or after growth had just started, approximately 1 m $\gamma$  of PGA was present in each tube. After 12 to 13 hours, 12 to 30 m $\gamma$  of PGA were present in the cultures started with 50 to 500 m $\gamma$  of PABA. The 12 m $\gamma$  of PGA formed from 50 m $\gamma$  of PABA in 12 to 13 hours were the same as that synthesized in the longer incubation periods (Table III).

The replacement of acid-hydrolyzed casein (Difco vitamin-free casamino acids) by an amino acid mixture or by an enzymatic digest of casein (14) decreased the amount of free PGA synthesized from PABA (Table III). The small decrease with the amino acid medium (C) may have been due in part to the reduction from 10 gm. of acid-hydrolyzed casein to 6.2 gm. of amino acids per liter. However, 10 gm. of the enzymatically digested casein were employed in Medium B, which gave the lowest values for PGA synthesis. This medium was modified from that of the acid-hydrolyzed casein medium (A) only by the omission of added tryptophan. The total sodium acetate concentration was kept the same. All three media shown

TABLE III  
Effect of Culture Medium and Time of Incubation upon Synthesis of Free Pteroylglutamic Acid from *p*-Aminobenzoic Acid by *Lactobacillus arabinosus*

Medium	PABA added	Free PGA in cultures after various times of incubation		
		20 hrs.	65 hrs.	90 hrs.
	mγ	mγ	mγ	mγ
A. Acid-hydrolyzed casein	50	15	11	12
	100	33	26	30
	200	80	68	80
	500	120	140	128
B. Enzymatically digested casein	50	5	5	6
	100	12	13	18
	200	11	12	18
	500	12	12	18
C. Amino acid mixture	50	7	8	
	100	16	18	
	200	60	60	
	500	60	90	

in Table III provided good growth for *Lactobacillus arabinosus* and similar response to graded amounts of PABA (almost maximal acid production with 2 mγ of PABA per tube). The decrease in PGA synthesis with the enzymatically digested casein might have been due to the presence of bound glutamic acid (strepogenin (15)) in contrast to the free glutamic acid in the other media. Any bound PGA which may have been formed would not have been detected in the present experiments. The addition of 2 gm. of L-glutamic acid per liter to the enzymatically digested casein medium did not alter the amounts of free PGA synthesized.

*Substitution of Pteric Acid Derivatives for PABA As Growth Factors for Lactobacillus arabinosus*—The PGA and pteric acid used in the present experiments were analyzed for free arylamine (11) and found to contain 2.2

and 1 per cent, respectively, when calculated as PABA. This must be taken into account in evaluating the growth-promoting effect of the pterioic acid compounds on *L. arabinosus*. The free arylamine, as measured above, may also include PABG. This compound was found to be practically as effective as PABA (on a molecular basis) as a growth factor for *L. arabinosus* when grown for 65 hours under the same conditions used for the pterioic acid and PGA experiments.

TABLE IV

*Utilization of Pteroylglutamic Acid for Growth of Lactobacillus arabinosus in Terms of Equivalent p-Aminobenzoic Acid Activity (65 Hour Incubation)*

PGA* added	Free PABA* of sample	Autoclaved with medium	Autoclaved separately	Sterilized by filtration	Autoclaved with medium	Autoclaved separately	Sterilized by filtration
		PABA activity			PGA activity†		
mγ	mγ	mγ	mγ	mγ	per cent	per cent	per cent
5	0.11	1.0	0.8	1.0	57	45	57
10	0.22	1.1	0.9	1.1	28	22	28
20	0.44	1.1	1.1	1.1	11	11	11
40	0.88	1.0	1.4	1.4	1	4	4
80	1.76	1.6	1.6	1.6	0	0	0
2	0.04	0.3	0.5	0.4	33	59	46
5	0.11	0.8	0.8	0.8	45	45	45
10	0.22	1.0	1.0	1.0	25	25	25
20	0.44	1.2	1.4	1.2	12	15	12
40	0.88	1.5	1.4	1.3	5	4	3

\* The PGA used in these experiments contained 2.2 per cent of free arylamine (PABA).

† Calculated on a molecular basis to express PGA activity relative to its PABA content after correction for free PABA. PGA contains 31.1 per cent bound PABA.

Activities of PGA and pterioic acid for *Lactobacillus arabinosus*, expressed in terms of equivalent PABA activity, are shown in Tables IV and V. The free PABA (or PABG) calculated from the analyses given above is also presented for each level of PGA or pterioic acid. Since there may have been further liberation of PABA or PABG in autoclaving PGA or pterioic acid with the medium, aliquots of the test material at pH 6.9 were autoclaved separately or sterilized by filtration and then added to the tubes prior to inoculation. The results in Table IV show no significant differences in the three types of test for PGA. However, pterioic acid which had been autoclaved promoted slightly more growth than that which had been filtered (Table V).

There was a decrease in the per cent of utilization of PGA and of pterioic

acid for growth of *Lactobacillus arabinosus* as the amounts tested were increased. With the larger amounts of PGA (Table IV) the growth obtained was almost completely accounted for by the calculated free PABA of the sample. At lower levels of PGA, the free PABA contributed a much smaller portion to the growth effect. In all cases PGA was at most only partially utilized as a substitute for, or as a source of, PABA for *L. arabinosus*, as shown by the per cent activity of PGA after correcting for free PABA. In similar experiments with pteric acid (Table V) the utilization for growth was a little higher on a molecular basis than that found for PGA.

TABLE V  
Utilization of Pteric Acid for Growth of *Lactobacillus arabinosus* in Terms of Equivalent *p*-Aminobenzoic Acid Activity (65 Hour Incubation)

Pteric* acid added	Free PABA* of sample	Autoclaved with medium	Autoclaved separately	Sterilized by filtration	Autoclaved with medium	Autoclaved separately	Sterilized by filtration
PABA activity					Pteric acid activity†		
mγ	mγ	mγ	mγ	mγ	per cent	per cent	per cent
1	0.01	0.20	0.40	0.20	43	89	43
2	0.02	0.32	0.70	0.28	34	77	30
5	0.05	0.46	0.87	0.40	19	37	16
10	0.1	0.46	0.93	0.65	8	19	13
20	0.2	0.97	1.3	0.85	9	13	7
40	0.4	1.9	1.3	1.1	8	5	4
80	0.8	Maximum	Maximum	1.2			1

\* The pteric acid used in these experiments contained 1 per cent of free arylamine (PABA).

† Calculated on a molecular basis to express pteric acid activity relative to its PABA content after correction for free PABA. Pteric acid contains 43.8 per cent bound PABA.

These experiments suggest the possibility that PGA and pteric acid may be split to PABA before utilization by *L. arabinosus*.

With 40 to 80 mγ of PGA (containing 1 to 2 mγ of free PABA) only one-half to three-quarters of maximal acid production was obtained in 65 hours with *Lactobacillus arabinosus*.<sup>4</sup> The 1 to 2 mγ of free PABA present in these amounts of PGA could account for this growth (Table IV). Maximal acid production was obtained with 80 mγ of pteric acid containing 0.8 mγ of free PABA (Table V). This would require utilization of only 3 to 4 per cent of the PABA bound in the pteric acid (in addition to the free PABA of the sample).

<sup>4</sup> In a personal communication, Dr. J. O. Lampen reports that PGA samples containing 0.2 to 1.0 per cent of free arylamine (PABA or PABG) had 2 to 3.5 per cent of the activity of PABA for *Lactobacillus arabinosus*. About 100 mγ of these preparations per 10 ml. afforded maximal acid production.

*Effect of PABA upon Utilization of PGA and Thymine by Lactobacillus casei*—Experiments which are not shown in tabular form have demonstrated that PABA (10 to 100  $\gamma$  per tube) could not replace PGA as a growth factor for *L. casei* and had no effect upon the utilization of maximal or suboptimal amounts of PGA by *L. casei* for acid production in 65 hours. Thymine partially replaced PGA as a growth factor for *L. casei* (1  $\gamma$  of thymine equals 0.16 m $\gamma$  of PGA) and, when present in excess, thymine supported half maximal acid production. This confirms other findings (12, 13). The addition of PABA did not affect acid production by *L. casei* in the presence of suboptimal or excess amounts of thymine.

#### DISCUSSION

PABA is an essential growth factor for *Lactobacillus arabinosus* (16, 17) and can be used in the synthesis of PGA. In the present experiments PGA replaced PABA to a very small extent for growth of *L. arabinosus*. PGA was also unable to replace PABA as a growth factor for a mutant strain of *Escherichia coli* (18). This suggests that PABA has other functions beside its use as a precursor of PGA and that conjugates of PABA may have to be split to free PABA before utilization by organisms which require PABA. The inability of PGA to overcome sulfonamide inhibition in organisms which do not require preformed PGA, e.g. *Escherichia coli*, *Staphylococcus aureus*, and *Diplococcus pneumoniae* (8), supports these conclusions. Under certain conditions sulfonamide action may inhibit the synthesis of PGA from PABA (8). This was shown with *Streptococcus faecalis* (Ralston) with a medium which permitted slow growth of the organism and in which PABA or pterioic acid compounds were stimulatory (8). The antagonism of sulfonamides by methionine and purines (19, 20) in addition to PABA indicates that these compounds also may be involved in or are products of some metabolic functions of PABA.

In the present experiments PABA and PABG have been found to be comparable in their growth-promoting effect for *Lactobacillus arabinosus* after 65 hours. Lampen reports<sup>5</sup> that after 24 hours PABG has only one-fourth the activity of PABA for this organism. Williams has shown that PABG has only one-twentieth of the antisulfanilamide activity of PABA for *Lactobacillus arabinosus* in 24 hour growth experiments (21). This may also indicate differences in the use of free PABA and its conjugates.

#### SUMMARY

The growth of *Lactobacillus arabinosus* in the presence of excess *p*-aminobenzoic acid is accompanied by the synthesis of pteroylglutamic acid. The free pteroylglutamic acid reaches a maximum after about 20 hours and is

<sup>5</sup> Personal communication.



present mainly in the culture medium. The source of amino acids in the medium markedly influences the amount of free pteroylglutamic acid formed.

Pteroylglutamic acid or pteric acid can only partially replace *p*-aminobenzoic acid as a growth factor for *Lactobacillus arabinosus*. This may depend upon the ability of the organism to liberate *p*-aminobenzoic acid from these conjugates. The findings also suggest that organisms which require *p*-aminobenzoic acid use it for other functions in addition to the synthesis of pteroylglutamic acid.

Excess *p*-aminobenzoic acid has no effect upon the utilization of thymine or of pteroylglutamic acid by *Lactobacillus casei*.

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# THE DISTRIBUTION AND EXCRETION OF PLUTONIUM ADMINISTERED INTRAVENOUSLY TO THE RAT\*

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Large scale processing of plutonium was necessary in the development and production of the atomic bomb. The future will undoubtedly see the development of other uses for this material. The toxic nature of plutonium has made the study of its metabolism in the animal organism a matter of great importance. Extensive animal experimentation is imperative if a full understanding of the toxic properties of plutonium is to be gained and if suitable means are to be devised for the protection of the health of persons working with this material.

The purpose of the experiments conducted at this laboratory was to study the effect of citrate ion concentration, valence state, and various other factors on the body distribution and excretion of plutonium following its intravenous administration to the rat.

## EXPERIMENTAL

*General Procedure*—Young male rats ranging in size from 200 to 250 gm. were used for the experiment. The animals were arranged in four groups of twelve rats each. Each animal was injected intravenously by way of the femoral vein with approximately 15  $\gamma$  of plutonium. In each case the volume of solution injected was 0.35 ml. The rats in the first group received plutonium in the form of a solution of  $\text{PuCl}_3$ , the second group received  $\text{Pu}(\text{NO}_3)_4$ , the third group received  $\text{PuO}_2(\text{NO}_3)_2$ , and the fourth group received  $\text{Pu}^{++++}$ -citrate ion complex in a 0.3 per cent solution of  $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 5\frac{1}{2}\text{H}_2\text{O}$ .

The actual amount of plutonium injected was determined by making "dummy" injections as follows: During the injection of a particular group of rats an amount of solution equivalent to that administered to each rat was discharged from the syringe into each of five 50 ml. volumetric flasks. After dilution to exactly 50 ml. with 4 N HCl, the solutions were assayed for plutonium by counting a suitable aliquot of each in a proportional  $\alpha$ -counter. The average of the five dummy injections was taken as the

\* Report written by Wright Langham.

average dose of plutonium received by the animals in the group. The animals were housed in metabolism cages that were especially constructed to facilitate the collection of urine and feces separately. Urine and feces samples were collected at 24 hour intervals through the 16th day, after which they were collected at 48 hour intervals. Each sample consisted of the pooled excretion from three rats.

In order to determine body deposition of the injected plutonium, one-third of the animals in each group was killed and examined at one of the following time intervals: 4, 16, 32, and 48 days after injection. Liver, kidneys, spleen, and carcass were saved separately for analysis.

*Methods of Analysis*—The urine, kidneys, liver, and spleen were prepared for analysis in essentially the same manner. The material was transferred to a 300 ml. Kjeldahl flask and digested with concentrated  $\text{HNO}_3$  and 30 per cent  $\text{H}_2\text{O}_2$  until a white ash remained. The ash was dissolved in 4 N HCl and the solution transferred to a volumetric flask, made up to volume, and a suitable aliquot analyzed for plutonium.

The carcass, consisting of skeleton and "balance,"<sup>1</sup> was dried and then ashed by heating in a muffle furnace at 450–500° for about 24 hours.

After cooling, the white flaky ash of the balance was separated from the skeleton by means of a 20 mesh sieve.

The skeleton and the ash from the balance were dissolved separately by repeated extractions with hot HCl. Suitable aliquots of these solutions were taken for plutonium analysis.

Feces samples were dried, weighed, and ground. An aliquot of the ground sample was transferred to a platinum crucible and ignited at a dull red heat. The grayish white ash was extracted several times with hot 4 N HCl. The residue was treated with HF and again extracted with 4 N HCl. The extracts were combined and made up to volume. A suitable aliquot of the feces ash solution was analyzed for plutonium.

#### RESULTS AND DISCUSSION

*Effect of Valence State and Citrate Ion on Urinary and Fecal Excretion of Plutonium Administered Intravenously to the Rat*—Figs. 1 to 4 present graphically the data showing the daily urinary and fecal excretion of plutonium when administered intravenously to the rat as  $\text{PuCl}_3$ ,  $\text{Pu}(\text{NO}_3)_4$ ,  $\text{PuO}_2(\text{NO}_3)_2$ , and  $\text{Pu}^{++++}$ -citrate complex. The most obvious point indicated by these results was the extremely high excretion of  $\text{PuO}_2^{++}$  in the urine during the 1st day. During the 1st day the urinary excretion of plutonium administered as  $\text{PuO}_2(\text{NO}_3)_2$  was 7.5 per cent of the injected dose as compared to 0.33, 0.57, and 0.71 per cent for  $\text{Pu}^{+++}$ ,  $\text{Pu}^{++++}$ , and

<sup>1</sup> All the carcass except skeleton, kidney, spleen, and liver.

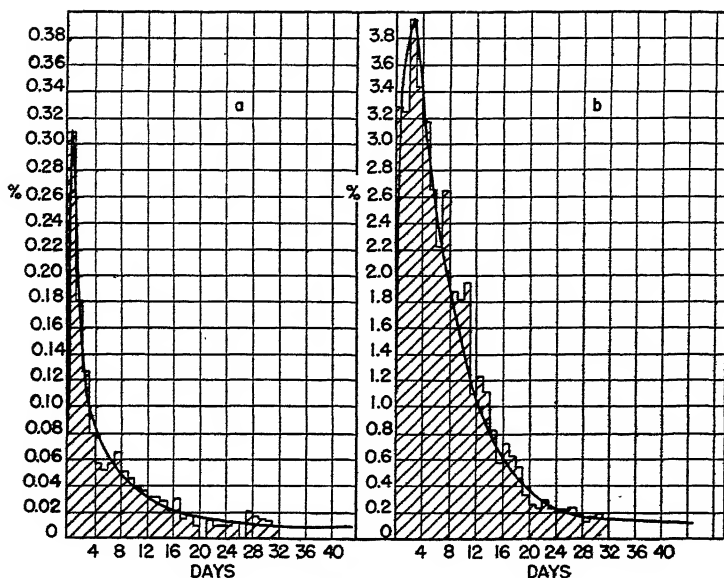


FIG. 1. Respective urinary (a) and fecal (b) excretion of plutonium following the intravenous administration of 15.2  $\gamma$  of Pu as  $\text{PuCl}_3$ . Per cent of dose excreted per day.

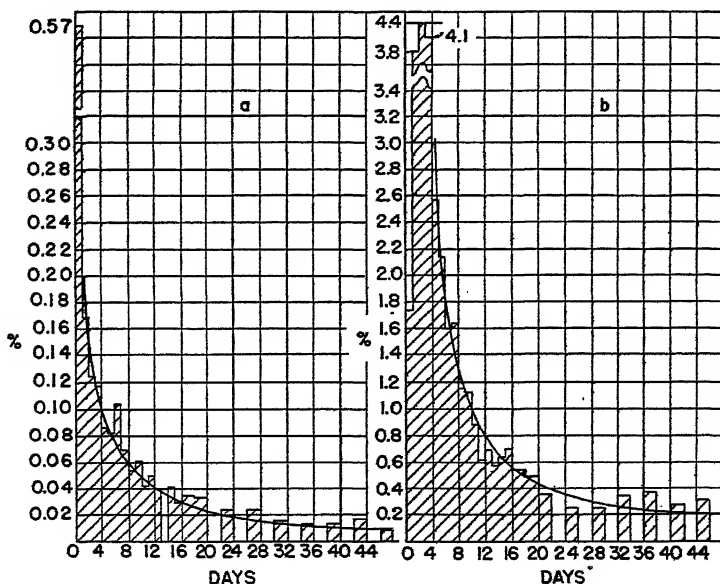


FIG. 2. Respective urinary (a) and fecal (b) excretion of plutonium following the intravenous administration of 14.2  $\gamma$  of Pu as  $\text{Pu}(\text{NO}_3)_4$ . Per cent of dose excreted per day.

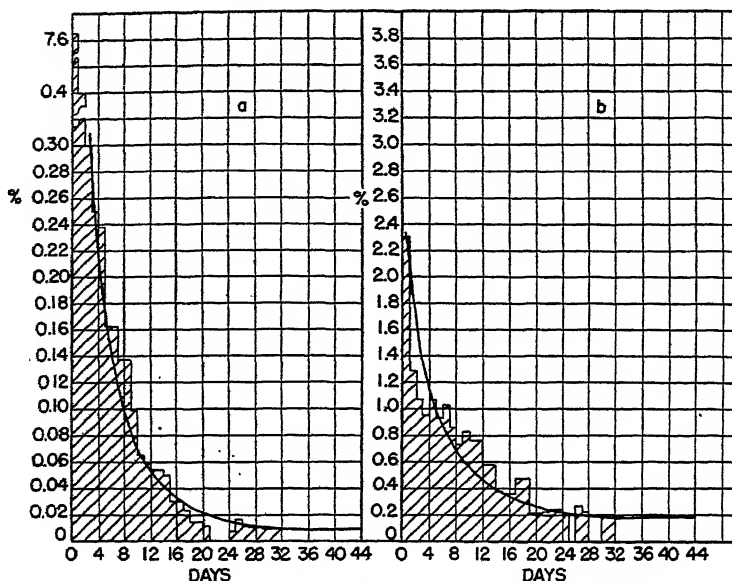


FIG. 3. Respective urinary (a) and fecal (b) excretion of plutonium following the intravenous administration of 15.1  $\gamma$  of Pu as  $\text{PuO}_2(\text{NO}_3)_2$ . Per cent of dose excreted per day.

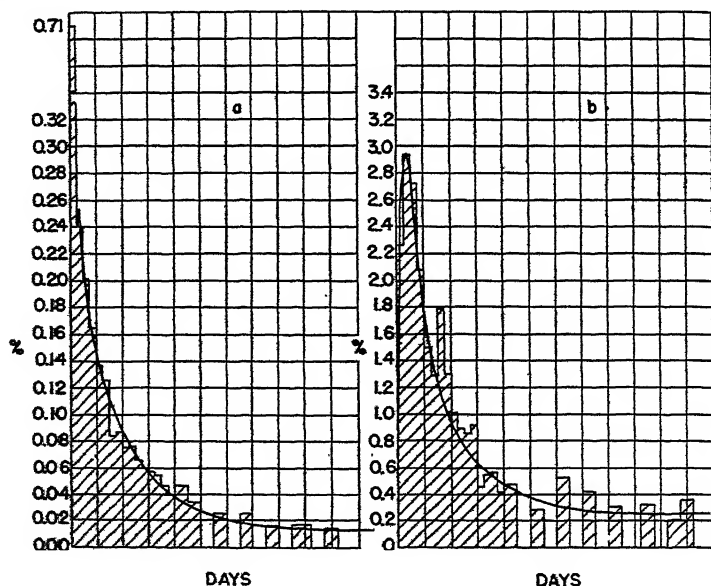


FIG. 4. Respective urinary (a) and fecal (b) excretion of plutonium following the intravenous administration of 15.0  $\gamma$  of Pu as  $\text{Pu}^{4+}$ -citrate complex. Per cent of dose excreted per day.

$\text{Pu}^{++++}$ -citrate complex respectively. Throughout the first 4 to 6 days the urinary excretion of plutonium injected as  $\text{PuO}_2(\text{NO}_3)_2$  remained somewhat higher than the excretion when the plutonium was injected in the other forms. This time interval may be assumed to be the time required for all of the  $\text{PuO}_2^{++}$  ions to be reduced to a lower valence. The very high urinary excretion of  $\text{PuO}_2^{++}$  was accompanied by a lowered fecal excretion rate. Only 5.6 per cent of the injected dose was excreted in the feces during the first 4 days as compared to 13.8 per cent for  $\text{PuCl}_3$ , 14.0 for  $\text{Pu}(\text{NO}_3)_4$ , and 10 per cent for  $\text{Pu}^{++++}$ -citrate complex. The high fecal excretion of plutonium after injection as  $\text{PuCl}_3$  or  $\text{Pu}(\text{NO}_3)_4$  may be, however, a result of its higher deposition in the liver (see Table II).

The data in Table I compare the urinary and fecal excretion of the plutonium at 1 day and at 30 days after injection. Table I was prepared by taking from the excretion curves shown in Figs. 1 to 4 what seemed to

TABLE I

*Comparison of Fecal and Urinary Excretion of Various Forms of Plutonium on 1st and 30th Days after Intravenous Administration to Rats*

Compound and valence	Per cent dose excreted 1st day			Per cent dose excreted 30th day*		
	Urine	Feces	$\frac{\text{Feces}}{\text{Urine}}$	Urine	Feces	$\frac{\text{Feces}}{\text{Urine}}$
$\text{PuCl}_3$	0.33	3.27	10:1	0.011	0.15	14:1
$\text{Pu}(\text{NO}_3)_4$	0.57	3.80	7:1	0.016	0.28	17:1
$\text{PuO}_2(\text{NO}_3)_2$	7.50	2.30	0.3:1	0.011	0.18	16:1
$\text{Pu}^{++++}$ -citrate	0.71	2.25	3:1	0.016	0.28	17:1

\* These values were taken from excretion curves shown in Figs. 1 to 4.

be an average excretion value 1 day and at 30 days after injection. The ratio of fecal to urinary excretion during the 1st day emphasizes the extent to which the excretion of  $\text{PuO}_2^{++}$  differed from the excretion of plutonium administered in other forms. During the 1st day the ratio was only 0.3:1 compared to 3:1 to 10:1 for the other forms. On the 30th day after injection there were no outstanding differences in either urinary or fecal excretion, regardless of the form in which the plutonium had been administered. At this time the over-all average daily urinary excretion was 0.014 per cent of the injected dose. The average fecal excretion was 0.22 per cent and the ratio of average fecal to urinary excretion was 16:1. These data emphasize the extremely high fecal excretion of plutonium by the rat. Regardless of the form in which the plutonium was injected, the fecal output 30 days after injection was 16 times as great as that in the urine. In this respect the human and the rat appear to differ widely.

The ratio of fecal to urinary excretion of plutonium by the human at 30 days is at most 1:1 (unreported data obtained at this laboratory). The wide difference in the rate at which the rat and the human excrete plutonium in the feces should be considered in an evaluation of toxicological studies in which the rat is used as the test animal.

TABLE II  
*Effect of Valence State, Citrate Ion, and Time after Injection on Metabolism of Plutonium Administered Intravenously to Rats*

Tissue or excretion	Days after injection	Per cent of injected dose recovered*				Days after injection	Per cent of injected dose recovered*			
		PuCl <sub>3</sub>	Pu(NO <sub>3</sub> ) <sub>4</sub>	Pu <sup>+++</sup> -citrate†	PuO <sub>2</sub> (NO <sub>3</sub> ) <sub>2</sub>		PuCl <sub>3</sub>	Pu(NO <sub>3</sub> ) <sub>4</sub>	Pu <sup>+++</sup> -citrate†	PuO <sub>2</sub> (NO <sub>3</sub> ) <sub>2</sub>
Liver.....	4	22.92	39.69	9.56	9.11	32	5.38			2.78
Spleen.....		0.73	1.19	0.67	0.51		0.73			0.33
Kidneys.....		2.20	1.36	1.64	1.91		0.50			0.73
Skeleton.....		44.91	29.43	56.93	56.54		51.81			56.84
Balance.....		13.54	11.88	13.70	11.21		7.86			9.46
Urine.....		0.90	0.93	1.34	7.89		1.20			9.80
Feces.....		17.15	15.11	10.89	5.71		33.86			20.54
Total.....		102.3	99.6	94.7	92.9		101.3			99.9
Liver.....	16	7.04	26.14	4.17	3.37	48	21.84	2.70		
Spleen.....		0.96	1.40	0.60	0.48		1.43	0.57		
Kidneys.....		0.67	0.74	0.79	1.14		0.43	0.36		
Skeleton.....		42.28	30.88	60.30	58.50		31.38	60.45		
Balance.....		11.95	7.28	10.15	7.58		5.21	7.85		
Urine.....		1.35	1.69	2.15	9.18		2.15	2.85		
Feces.....		37.61	26.03	20.33	12.91		39.65	29.68		
Total.....		101.9	94.2	99.0	93.2		102.1	104.5		

Each value is an average of results obtained from the tissues or excrement of three rats for PuCl<sub>3</sub>, four rats for Pu(NO<sub>3</sub>)<sub>4</sub> and Pu<sup>+++</sup>-citrate, and six rats for PuO<sub>2</sub>(NO<sub>3</sub>)<sub>2</sub>.

\* The dosages injected were 15.2 γ of Pu as PuCl<sub>3</sub>, 14.2 γ as Pu(NO<sub>3</sub>)<sub>4</sub>, 15.0 γ as Pu<sup>+++</sup>-citrate, and 15.1 γ as PuO<sub>2</sub>(NO<sub>3</sub>)<sub>2</sub>.

† Pu<sup>+++</sup> in a 0.3 per cent solution of Na<sub>3</sub>C<sub>6</sub>H<sub>5</sub>O<sub>7</sub>·5½H<sub>2</sub>O.

There were no outstanding differences in the excretion of Pu<sup>+++</sup> when injected as the citrate complex and as Pu(NO<sub>3</sub>)<sub>4</sub>. In the former case, however, fecal excretion was somewhat lower during the first few days. The lower fecal excretion may be due to a lower deposition in the liver (see Table II).

*Effect of Valence State and Citrate Ion on Body Distribution of Plutonium Administered Intravenously to the Rat*—The data in Table II show the distribution of plutonium in the rat body at 4, 16, 32, and 48 days after intravenous injection as  $\text{PuCl}_3$ ,  $\text{Pu}(\text{NO}_3)_4$ ,  $\text{Pu}^{++++}$ -citrate complex, and  $\text{PuO}_2(\text{NO}_3)_2$ . Regardless of the original form in which the plutonium was injected, the skeleton was a major site of deposition. When the plutonium was administered as  $\text{Pu}^{++++}$ -citrate and  $\text{PuO}_2(\text{NO}_3)_2$ , the skeletal deposition was approximately 60 per cent of the injected dose. 4 days following injection of plutonium as  $\text{PuCl}_3$  and  $\text{Pu}(\text{NO}_3)_4$  deposition in the skeleton was 44.9 and 29.4 per cent of the dose respectively.

Deposition of plutonium in the liver was low when it was injected in a form giving high deposition in the skeleton. 4 days following injection as  $\text{Pu}^{++++}$ -citrate and  $\text{PuO}_2(\text{NO}_3)_2$ , the amounts deposited in the liver were 9.6 and 9.1 per cent of the dose respectively. Deposition of plutonium in the liver 4 days after administration as  $\text{PuCl}_3$  was 22.9 per cent of the injected dose. Plutonium injected as  $\text{Pu}(\text{NO}_3)_4$  was deposited in the liver to the extent of 39.7 per cent of the injected dose.

No outstanding differences were observed in the deposition of plutonium in the kidney following intravenous injection of the various forms. At the end of the 4th day kidney deposition ranged from 1.36 to 2.20 per cent. At 16 days deposition in the kidney ranged from 0.67 per cent in the case of  $\text{PuCl}_3$  to 1.14 per cent for  $\text{PuO}_2(\text{NO}_3)_2$ .

Deposition of plutonium in the spleen following intravenous injection was not greatly different for the various forms of plutonium injected. As was the case with the liver, the highest deposition in the spleen was obtained with  $\text{Pu}(\text{NO}_3)_4$ .

The time intervals chosen in this study (4, 16, 32, and 48 days) were much too short to permit any significant conclusions regarding the effect of time on distribution and relocation of plutonium in the body following intravenous injection in various forms.

In general the plutonium content of the liver, kidneys, and balance decreased with time. The skeleton and spleen did not show any significant changes during the short time intervals allowed for the experiment.

The data in Table II and the excretion data shown in Figs. 1 to 4 suggest that fecal excretion of plutonium was higher for those forms giving higher deposition in the liver, namely  $\text{PuCl}_3$  and  $\text{Pu}(\text{NO}_3)_4$ .

*Effect of Size of Dose on Metabolism of Plutonium in the Rat*—If urinary excretion is to provide a basis for determining the degree of exposure of workers to plutonium, the metabolism of this material in the body must be independent of the size of dose; i.e., the metabolism must be essentially the same whether the body content results from a single large dose or from



several small ones. The following experiment was devised to study the effect of size of dose on the excretion and body distribution of plutonium when administered intravenously to the rat.

Groups of mature male rats were injected with 0.032, 1.1, 5.3, 15.0, and 52  $\gamma$  of plutonium respectively. The plutonium was administered as  $\text{Pu}^{++++}$ -citrate complex in a solution 0.5 per cent with sodium citrate. The pH of the solution was approximately 6 and the volume injected was kept constant, 0.35 ml. Urine and feces were collected daily for 6 days and analyzed for plutonium. At the close of the 6th day following injection the animals were sacrificed and their tissues analyzed for plutonium.

TABLE III

*Daily Excretion of Intravenously Administered Plutonium in Relation to Size of Dose (Rat)*

Injected as  $\text{Pu}^{++++}$ -citrate complex prepared from  $\text{Pu}(\text{NO}_3)_4$  by making the solution 0.5 per cent with  $\text{Na}_2\text{C}_6\text{H}_5\text{O}_7 \cdot 5\frac{1}{2}\text{H}_2\text{O}$ . The pH of each solution was approximately 6. Each value is the average of results from three rats, except for the 15  $\gamma$  dose, for which each value is an average of results from twelve rats.

Period after injection	Size of dose and per cent of dose excreted								
	0.032 $\gamma$	1.1 $\gamma$		5.3 $\gamma$		15 $\gamma$		52 $\gamma$	
days	Urine	Urine	Feces	Urine	Feces	Urine	Feces	Urine	Feces
0.0-0.5	0.609	0.665	1.74	0.467	0.85	0.436	0.38	0.733	2.06
0.5-1	0.312	0.218	0.88	0.476	1.57	0.269	1.89	0.189	1.11
1 -2	0.338	0.265	2.04	0.419	2.51	0.240	2.94	0.321	2.13
2 -3	0.276	0.133	1.37	0.220	1.71	0.201	2.72	0.244	1.89
3 -4	0.181	0.151	0.81	0.165	1.13	0.165	2.07	0.208	1.42
4 -5	0.176	0.139	0.80	0.104	1.30	0.137	1.50	0.199	1.40
5 -6	0.176	0.139	0.80	0.104	1.30	0.126	1.29	0.199	1.40
Total.....	2.07	1.71	8.44	1.96	10.37	1.57	12.79	2.09	11.41

The data in Table III show the relationship between size of dose and the daily urinary and fecal excretion of plutonium injected intravenously into the rat. The *per cent* of the injected dose excreted per day in both feces and urine was not significantly affected by the size of the dose. In fact, the agreement in per cent excreted for the various sized doses was remarkably close. At the end of the 6th day after injection the spread in total fecal excretion was from 8.4 to 12.8 per cent of the dose. The spread in total urinary excretion was from 1.6 to 2.1 per cent.

The distribution of plutonium in the animal body was likewise unaffected by the size of the injected dose (Table IV). The skeleton was the principal

site of deposition. The results for skeletal deposition ranged from 56.1 to 60.5 per cent for all doses.

The per cent of the dose present in the total blood volume was also quite constant. The range in blood content for the various dosages 6 days following injection was 1.0 to 1.5 per cent. The amount of plutonium deposited in the liver ranged from 7.8 to 10.4 per cent.

*Metabolism of Plutonium Administered Orally to the Rat*—Five groups of animals consisting of three young male rats each were selected for the experiment. Groups 1, 2, and 3 were given 46, 198, and 488  $\gamma$  of  $\text{Pu}^{++++}$ , respectively. The plutonium was administered in 5 per cent sodium citrate

TABLE IV

*Effect of Size of Dose on Metabolism of Plutonium Administered Intravenously to Rats*

Injected as  $\text{Pu}^{++++}$ -citrate complex prepared from  $\text{Pu}(\text{NO}_3)_4$  by making the solution 0.5 per cent with  $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 5\frac{1}{2}\text{H}_2\text{O}$ . The pH of each solution was approximately 6. All animals were sacrificed at the end of the 6th day. Each value is the average of results from three rats. Values for the 15  $\gamma$  dose are an average of results from twelve rats.

Organ or excretion	Size of injected dose and per cent of dose per organ				
	0.032 $\gamma$	1.1 $\gamma$	5.3 $\gamma$	15 $\gamma$	52 $\gamma$
Liver.....	7.79	9.32	10.43	9.56	9.39
Spleen.....	0.66	0.44	0.45	0.67	0.49
Kidneys.....	1.33	1.22	1.22	1.64	1.46
Skeleton.....	56.12	57.30	60.46	56.93	60.38
Balance.....	18.34	14.30	14.39	13.70	14.31
Blood.....	1.50	1.25	1.00		1.10
Urine.....	2.07	1.71	1.95	1.58	2.09
Feces.....	9.13	8.45	10.37	12.79	11.41
Total.....	96.9	94.0	100.3	96.9	100.6

solution. Group 4 was given 2000  $\gamma$  of  $\text{Pu}^{++++}$  in 0.5 per cent sodium citrate. The last group was given 488  $\gamma$  of  $\text{Pu}^{++++}$  in the absence of any sodium citrate. All doses were administered by giving one-fifth of the total amount of plutonium per day for 5 consecutive days. All animals were sacrificed 4 days after the last administration of plutonium. Livers, skeleton, and balance were analyzed separately for plutonium.

The results are given in Table V. These results indicate that absorption of plutonium from the gastrointestinal tract (in the absence of citrate ion) was quite low, 0.01 per cent. Absorption was increased by the simultaneous administration of a large excess of sodium citrate. The increase in absorp-

tion, however, was not great. Only about 0.3 per cent of the plutonium was absorbed when given in a 5 per cent solution of  $\text{Na}_2\text{C}_6\text{H}_5\text{O}_7 \cdot 5\frac{1}{2}\text{H}_2\text{O}$ .

The amount of plutonium administered, and the strength of the sodium citrate solution used did not significantly affect the per cent of the absorbed dose deposited in the liver and skeleton. If the amount of absorbed plutonium that was excreted in the urine and feces during the time of the experiment is disregarded, the average liver deposition for all dosages was 7.3 per cent of the amount absorbed. The average skeletal deposition under these conditions was 78.9 per cent of the absorbed material. These results indicate that plutonium absorbed slowly into the system, as from

TABLE V

*Absorption and Body Distribution of Plutonium Administered Orally to Rats*

All doses were administered by giving one-fifth of the total amount of plutonium per day for 5 consecutive days. The amount of the absorbed plutonium excreted in feces and urine during the experimental period was neglected. The animals were sacrificed 10 days after the beginning of the experiment.

Total doses of Pu	Concentration, $\text{Na}_2\text{C}_6\text{H}_5\text{O}_7 \cdot 5\frac{1}{2}\text{H}_2\text{O}$	Dose absorbed	Per cent of absorbed Pu per organ		
			Liver	Skeleton	Balance
$\gamma$	per cent	per cent			
46	5	0.34	7.8	70.8	21.4
198	5	0.32	8.2	81.7	10.1
488	5	0.25	3.7	88.1	8.2
488	0	0.01	7.5	80.0	12.5
2000	0.5	0.06	9.4	74.2	16.3
Average.....			7.3	78.9	13.7

the gastrointestinal tract, may result in a greater deposition in the skeleton than it does when administered intravenously even as  $\text{Pu}^{++++}$ -citrate complex or as  $\text{PuO}_2(\text{NO}_3)_2$ .

This paper is based on work performed under United States Government contract No. W-7405-Eng-36 at the Los Alamos Scientific Laboratory of the University of California.

## SUMMARY

1. The 1st day following intravenous injection of  $\text{PuO}_2(\text{NO}_3)_2$  the urinary excretion of plutonium was 7.5 per cent of the dose as compared to 0.33, 0.57, and 0.71 per cent when administered as  $\text{PuCl}_3$ ,  $\text{Pu}(\text{NO}_3)_4$ , and  $\text{Pu}^{++++}$ -citrate complex respectively. Fecal excretion during the 1st day was correspondingly lower following injection of  $\text{PuO}_2(\text{NO}_3)_2$ . On the 30th

day following intravenous injection there were no significant differences in either urinary or fecal excretion of plutonium administered as  $\text{PuCl}_3$ ,  $\text{Pu}(\text{NO}_3)_4$ ,  $\text{Pu}^{++++}$ -citrate complex, or  $\text{PuO}_2(\text{NO}_3)_2$ . At this time the average urinary excretion was 0.014 per cent of the injected dose and the average fecal excretion was 0.22 per cent. The average ratio of fecal to urinary excretion was 16:1.

2. The skeleton was a major site of deposition regardless of the form in which the plutonium was injected. 4 days following injection of plutonium as  $\text{PuCl}_3$ ,  $\text{Pu}(\text{NO}_3)_4$ ,  $\text{Pu}^{++++}$ -citrate complex, and  $\text{PuO}_2(\text{NO}_3)_2$  skeletal deposition was 44.9, 29.4, 56.9, and 56.5 per cent of the injected dose respectively. Deposition in the liver under the above conditions was 22.9, 39.7, 9.6, and 9.1 per cent of the injected dose respectively. Deposition of plutonium in kidney, spleen, and in balance was not greatly affected by the form in which the plutonium was administered.

3. The size of the injected dose of  $\text{Pu}^{++++}$ -citrate complex did not affect the per cent of the dose excreted in the feces and urine. Likewise, the size of dose did not alter the per cent of injected material present in the various tissues 6 days following injection.

4. In the absence of citrate ion absorption of plutonium from the gastrointestinal tract was quite low, 0.01 per cent of the administered dose. When  $\text{Pu}^{++++}$  was administered orally in 5 per cent sodium citrate solution, 0.3 per cent of the administered dose was absorbed.

5. 79 per cent of the plutonium absorbed from the gastrointestinal tract was deposited in the skeleton and 7.5 per cent in the liver. Plutonium absorbed slowly via this route seems to give a higher deposition in the skeleton than it does when administered intravenously as  $\text{PuCl}_3$ ,  $\text{Pu}(\text{NO}_3)_4$ ,  $\text{Pu}^{++++}$ -citrate complex, or  $\text{PuO}_2(\text{NO}_3)_2$ .



# AN X-RAY DIFFRACTION STUDY OF HIGH PHOSPHATE BONES\*

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PLATES 1 TO 3

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In recent publications, Sobel, Rockenmacher, and Kramer (1, 2) presented a study on the variation of composition of rat bones with variation of the relative concentrations of calcium, phosphorus, and bicarbonate in the blood serum, induced by different diets. In their experimental group on a high phosphorus-low calcium diet without vitamin D, the residual total base (expressed as calcium, which is about 95 per cent of the total base) to phosphorus ratio in the bones was 1.36 instead of the usual ratio of about 1.5. They expressed the composition of these bones empirically as  $[\text{CaHPO}_4]_{1.6}[\text{CaCO}_3][\text{Ca}_3(\text{PO}_4)_2]_{2.0}$  which would indicate a  $\text{CaHPO}_4$  content of about 23 per cent.

Although there is general agreement in the literature that the composition of bone may vary widely,\* most x-ray diffraction investigations indicate that the bone salts have an apatite or apatite-like structure (3-5). The present investigation is an x-ray diffraction study of high phosphate bones to detect possible changes in the structure of the bone salts, and particularly to seek physical evidence of the presence of  $\text{CaHPO}_4$  in these bones.

## Experiments on Bone

The rats used in this investigation were of the same breeding stock as those used by Sobel *et al.* (1, 2). Rats 23 to 25 days old were placed on a diet containing about 0.03 per cent calcium and 0.9 per cent phosphorus (Diet C (1)), with no vitamin D, and were kept in a dark room. Litter mates were placed on the breeding diet as normal controls. After the rats

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had subsisted on the diet for 1 month, they were sacrificed by cutting the blood vessels in the neck. The blood was collected, pooled, and the serum analyzed for calcium and phosphorus. The average calcium value was 5.5 mg. per cent and the average phosphorus value was 10 mg. per cent, agreeing generally with the values reported by Sobel *et al.* (2) for rats on this diet. The femurs and tibias were removed from the freshly killed animals, cleaned of adhering tissue, slit open, and soaked in two changes of alcohol and one of ether to remove fatty material. Bone analyses were in agreement with those reported previously for rats raised under almost identical conditions (1, 2).

The fat-extracted femurs were ground to a powder, and portions of each powder were ashed at 600° and at 900° for several hours. Powder diffraction patterns were made on small amounts of specimen contained in

TABLE I  
*Summary of x-Ray Diffraction Results on Bone*

Bone specimen	Treatment °C.	Predominant pattern	Weak pattern
High phosphate	None	Apatite	
	600	"	$\beta$ -Ca <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub>
	900	$\beta$ -Ca <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub>	Apatite
Normal	None	Apatite	
	600	"	
	900	"	

a thin walled Pyrex capillary, by means of a 57.3 mm. Debye-Scherrer camera and nickel-filtered copper K<sub>α</sub> radiation. Each exposure was 7 to 8 hours.

Fig. 1 shows the x-ray diffraction patterns of normal and high phosphate bone, before and after ignition. Table I summarizes the results. The most striking and significant feature is that the pattern of the high phosphate bone ashed at 900° is completely different from the others. This pattern represents the structure of  $\beta$ -tricalcium phosphate, whereas the other patterns are of the apatite type. The diffraction pattern of the high phosphate bone ashed at 600° is somewhat different from its normal counterpart, but the difference could be accounted for as being due to a transformation of some of the material to  $\beta$ -tricalcium phosphate at this temperature. No CaHPO<sub>4</sub>·2H<sub>2</sub>O was detected from the diffraction patterns of the unashed bone.  $\gamma$ - and  $\beta$ -calcium pyrophosphate, which would be formed from CaHPO<sub>4</sub> at 600° and 900° respectively (6), could not be detected from the diffraction patterns of bone ashed at 600° and 900°.

A chemical test for pyrophosphate was performed, based on its hydrolysis

to orthophosphate on heating in 1 N sulfuric acid solution for 15 minutes in a boiling water bath. Negative results were obtained with both the high phosphate and normal bones ashed at 900°, while a control containing pyrophosphate gave a positive test.

### *Experiments on Inorganic Mixtures*

In order to interpret these facts, similar experiments were done on mixtures of  $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$  and a commercial c.p. tertiary calcium phosphate

TABLE II

*Summary of x-Ray Diffraction Results on Inorganic Mixtures of Calcium Phosphates and Pure Materials*

Specimen	Treatment	Predominant pattern	Weak pattern
	°C.		
$\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$	None	$\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$	
	600	$\gamma\text{-Ca}_2\text{P}_2\text{O}_7$	
	900	$\beta\text{-Ca}_2\text{P}_2\text{O}_7$	
Hydroxyapatite (commercial c.p. tricalcium phosphate)	None	Apatite	
	900	"	
Mixture I. 8% $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ , 92% hydroxyapatite	None	"	$\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$
	600	"	$\gamma\text{-Ca}_2\text{P}_2\text{O}_7$
	900	"	$\beta\text{-Ca}_2(\text{PO}_4)_2$
Mixture II. 24% $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ , 76% hydroxyapatite	None	" and $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$	
	600	Apatite	$\gamma\text{-Ca}_2\text{P}_2\text{O}_7$
	900	$\beta\text{-Ca}_2(\text{PO}_4)_2$	Apatite
Mixture III. 40% $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ , 60% hydroxyapatite	None	$\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ and apatite	
	600	Apatite and $\gamma\text{-Ca}_2\text{P}_2\text{O}_7$	
	900	$\beta\text{-Ca}_2(\text{PO}_4)_2$	$\beta\text{-Ca}_2\text{P}_2\text{O}_7$

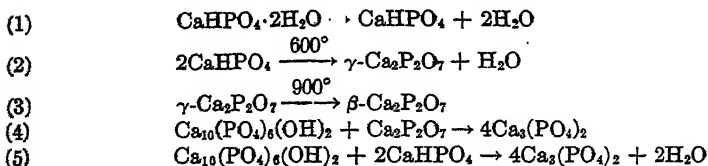
which showed an apatite diffraction pattern. The  $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$  was prepared in the laboratory and its diffraction pattern corresponded to that reported for this compound (7). A diffraction pattern of the ignited c.p. tertiary salt showed an apatite pattern; as there was no more than a trace of carbonate present, it was assumed to be hydroxyapatite. The mixtures consisted of (Mixture I) 8 per cent  $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$  and 92 per cent hydroxyapatite, (Mixture II) 24 per cent  $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$  and 76 per cent hydroxyapatite, and (Mixture III) 40 per cent  $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$  and 60 per cent hydroxyapatite. Portions of these mixtures were heated at 600° and at 900° for 2 hours. Diffraction patterns were made of all specimens. The results are shown in Figs. 2 and 3 and are summarized in Table II. Mixture II comes closest in composition to the high phosphate bone, and the



diffraction pattern of the ignited material most closely resembles those of ignited high phosphate bones.

The pyrophosphate test was performed on the ignited samples, as described for bone. Small amounts (about 5 per cent) of pyrophosphate were found in Mixtures I and II ignited at 600° and 900°, and large amounts in Mixture III ignited at 600° and 900°.

The reactions involved in the ignition may be written as



Apparently, any pyrophosphate that may be formed according to reaction (2) will react with the hydroxyapatite present according to reaction (4). A stoichiometric mixture for reaction (4) or (5) will contain initially 25.5 per cent  $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$  and 74.5 per cent hydroxyapatite.

#### DISCUSSION

In spite of the large amount of work done in the field, the mechanism of bone formation and its exact structure are still a matter of discussion. Shear and Kramer (8) suggested that the first step in the deposition of bone salts is the formation of  $\text{CaHPO}_4$ , because, if the ion product  $[\text{Ca}]^{++}[\text{HPO}_4]^-$  is much below the solubility product of  $\text{CaHPO}_4$ , calcification *in vivo* or *in vitro* will not take place. Logan and Taylor (9) showed that  $\text{CaHPO}_4$  was initially present in tertiary calcium phosphate precipitates at pH 7.3, but rapidly disappeared unless an excess of phosphate was present. Sobel, Rockenmacher, and Kramer (1, 2) performed what may be considered in part as an *in vivo* counterpart of Logan and Taylor's experiments. Among other things they were able to produce bones containing an excess of phosphate corresponding to a content of about 23 per cent  $\text{CaHPO}_4$ . However, the presence of  $\text{CaHPO}_4$  had never been directly demonstrated in bone either by chemical methods (10) or by x-ray diffraction (11, 12).

Morgulis (10) stated that any  $\text{CaHPO}_4$  present in bone would be converted to pyrophosphate on incineration. These authors could detect no such pyrophosphate, and this was considered as evidence against the  $\text{CaHPO}_4$  theory of bone formation. Our experiments on inorganic mixtures show that such conclusions are erroneous, because on heating to 900° any  $\text{CaHPO}_4$  that might be present in bone would first react with the apatite present to form tricalcium phosphate. Only amounts of  $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$  in excess of 26 per cent of the total would form pyrophosphate on

ignition. We could not detect any pyrophosphate in the ignited high phosphate bones containing empirically about 23 per cent  $\text{CaHPO}_4$ .

Our x-ray diffraction studies did not detect any  $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$  in the high phosphate bones, or any  $\text{CaHPO}_4$  or  $\gamma\text{-Ca}_2\text{P}_2\text{O}_7$  in these bones ignited at  $600^\circ$ . Mixture I, containing 8 per cent  $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$  and 92 per cent hydroxyapatite, gave diffraction patterns showing these lines (Fig. 2; Table II). Thus, any  $\text{CaHPO}_4$  present in the bones must be in a state of aggregation which gives rise to very weak or no x-ray diffraction patterns. This may be in the form of a finely divided state, which may or may not be adsorbed on the surface of the apatite crystallites, or may be included within the apatite lattice. If the  $\text{CaHPO}_4$  had a much more finely divided particle size than bone apatite, x-ray diffraction would not show it, as it is well known that extremely finely divided crystalline materials do not give clear diffraction patterns. Because of the small particle size of bone apatite ( $10^{-5}$  to  $10^{-6}$  cm.), still smaller particles would provide such powerful adsorption forces that the  $\text{CaHPO}_4$ , if evenly distributed, would greatly resemble adsorbed material, if not actually adsorbed. If the  $\text{CaHPO}_4$  were present adsorbed to the surface of the apatite crystallites, it would not be detected by x-ray diffraction, according to Walden and Cohen (13). If the  $\text{CaHPO}_4$  were a part of the apatite lattice, it would lose its own crystalline identity and not give its characteristic diffraction pattern. It was pointed out by Eisenberger, Lehrman, and Turner (4) that the apatite lattice is very susceptible to isomorphous substitution, and wide variations in composition may be sustained without change in structure.

It was observed that the diffraction patterns of the inorganic mixtures ignited at  $600^\circ$  do not show the partial conversion to  $\beta\text{-Ca}_3(\text{PO}_4)_2$  which the high phosphate bone undergoes at this temperature. In addition, chemical tests showed the presence of small amounts of pyrophosphate even in the presence of an excess of apatite in the inorganic mixtures after ignition at  $900^\circ$ , whereas no such pyrophosphate could be detected in bone. The difference may be due to the excess phosphate in the high phosphate bone being in much closer contact with the apatite than could be achieved by grinding the crystalline components with a mortar and pestle, and thereby reacting more readily. Close contact could be achieved either by adsorption of the  $\text{CaHPO}_4$  on the surface of the apatite or its inclusion within the apatite lattice. Because the apatite crystallites in bone are extremely small, large amounts of material may be adsorbed on the surface. Either explanation appears to be equally possible at this time.

$\beta\text{-Ca}_3(\text{PO}_4)_2$  was produced by heating  $\alpha$ -tricalcium phosphate<sup>1</sup> to  $900^\circ$

<sup>1</sup> The term  $\alpha$ -tricalcium phosphate is used by different authors to represent either the transformation product obtained by heating  $\text{Ca}_3(\text{PO}_4)_2$  at  $1700^\circ$  (4, 14), or a precipitated hydrated  $\text{Ca}_3(\text{PO}_4)_2 \cdot 2\text{H}_2\text{O}$  (5, 15). We are using this term in the latter sense.

(15-17). Hodge and coworkers considered this substance to be hydroxyapatite containing an excess of adsorbed phosphate, and that these combine on heating at  $900^{\circ}$  to form  $\beta\text{-Ca}_3(\text{PO}_4)_2$ . Dallemagne and coworkers (5, 15) disagree with this view-point, and consider the material to be  $\alpha$ -tricalcium phosphate. From our experiments, it would seem possible to produce  $\beta\text{-Ca}_3(\text{PO}_4)_2$  from hydroxyapatite and  $\text{CaHPO}_4$ , thus lending some support to Hodge's views, although there is no doubt that it could also be prepared from  $\alpha$ -tricalcium-phosphate.

In conclusion, it seems most probable that the inorganic matter of the high phosphate bones consists of apatite crystallites with  $\text{CaHPO}_4$  either adsorbed on the surface or included within the crystal structure.  $\text{CaHPO}_4$  apparently represents the first solid deposited on calcification, according to the theory of Shear and Kramer (8). Under the usual physiological concentrations of ions, this rapidly hydrolyzes to a tertiary calcium phosphate or apatite and the amount of  $\text{CaHPO}_4$  in normal bone at any given time may be very small. The concentrations of calcium in the serum of the rats from which the high phosphate bones were taken was only 5 to 6 mg. per cent, and, as much of this is in combination with the serum proteins, the amount of ionized calcium is very small. On the other hand, the concentration of inorganic phosphate was relatively high (9 to 10 mg. per cent). Precipitates in which the presence of  $\text{CaHPO}_4$  persisted after standing were obtained by Logan and Taylor (9) only from low calcium-high phosphate solutions in their studies of calcium phosphate precipitates. This may explain the appreciable amounts of  $\text{CaHPO}_4$  which are present in the high phosphate bones.

It is hoped that further studies (which are in progress), with x-ray diffraction, heat transformation, and chemical agents, of bones of different compositions and inorganic mixtures will throw more light on the structure of bone.

#### SUMMARY

1. X-ray diffraction studies reveal that high phosphate bones of the approximate empirical composition  $[\text{CaHPO}_4]_{1.5}[\text{Ca}_3(\text{PO}_4)_2]_{2.0}\text{CaCO}_3$  undergo a transformation at  $900^{\circ}$  from the apatite structure to  $\beta$ -tricalcium phosphate. At  $600^{\circ}$ , this transformation takes place partially. No  $\text{CaHPO}_4$  could be detected in unashed bone, nor could  $\gamma$ - or  $\beta$ -calcium pyrophosphate be found in bone ashed at  $600^{\circ}$  or at  $900^{\circ}$ .

2. Inorganic mixtures of  $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$  and hydroxyapatite when ignited at  $900^{\circ}$  will also form some  $\beta$ -tricalcium phosphate, depending on the relative amounts of each present. When  $\text{CaHPO}_4$  is present in excess, calcium pyrophosphate is also formed. These mixtures do not form any  $\beta$ -tricalcium phosphate at  $600^{\circ}$ , but show the diffraction lines of both apatite and  $\gamma\text{-Ca}_2\text{P}_2\text{O}_7$ .

3. It is concluded that  $\text{CaHPO}_4$  is present in the high phosphate bones, either adsorbed on the surface of the apatite crystallites or as an integral part of the lattice but not as an individual crystalline entity.

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## EXPLANATION OF PLATES

## PLATE 1

Fig. 1. X-ray diffraction patterns of normal and high phosphate bones. *A*, high phosphate bone; *A-1*, ashed at 600°; *A-2*, ashed at 900°. *B*, normal bone; *B-1*, ashed at 600°; *B-2*, ashed at 900°.

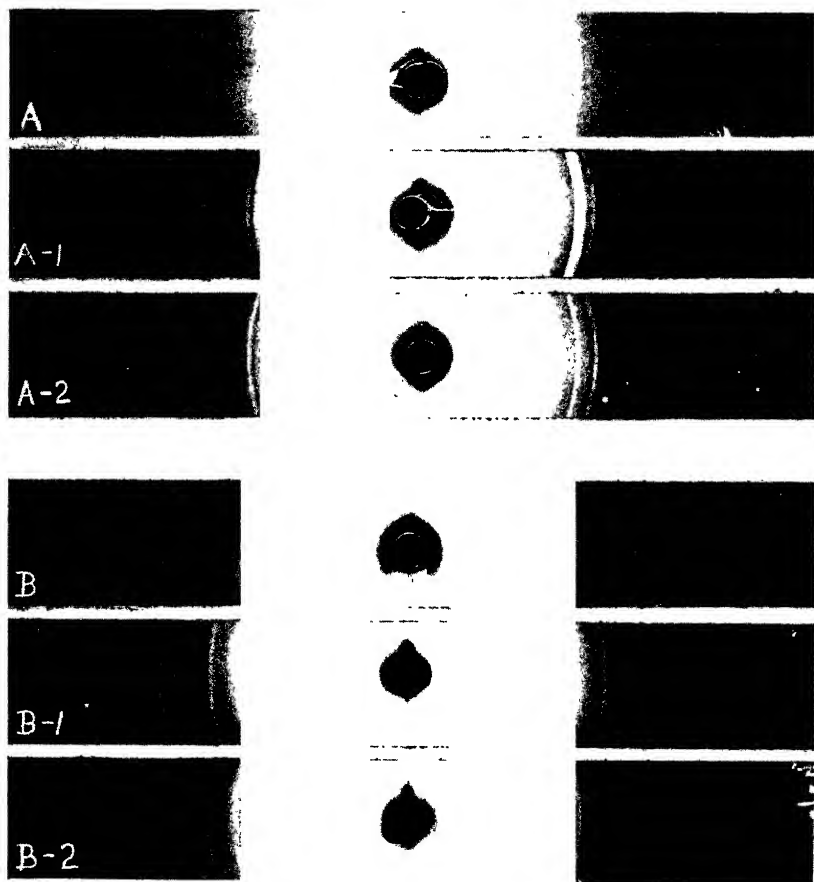
## PLATE 2

Fig. 2. X-ray diffraction patterns of inorganic mixtures. Mixture I, 8 per cent  $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ -92 per cent  $\text{Ca}_{10}(\text{OH})_2(\text{PO}_4)_6$ ; Mixture I-a, ashed at 600°; Mixture I-b, ashed at 900°. Mixture II, 24 per cent  $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ -76 per cent  $\text{Ca}_{10}(\text{OH})_2(\text{PO}_4)_6$ ; Mixture II-a, ashed at 600°; Mixture II-b, ashed at 900°. Mixture III, 40 per cent  $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ -60 per cent  $\text{Ca}_{10}(\text{OH})_2(\text{PO}_4)_6$ ; Mixture III-a, ashed at 600°; Mixture III-b, ashed at 900°.

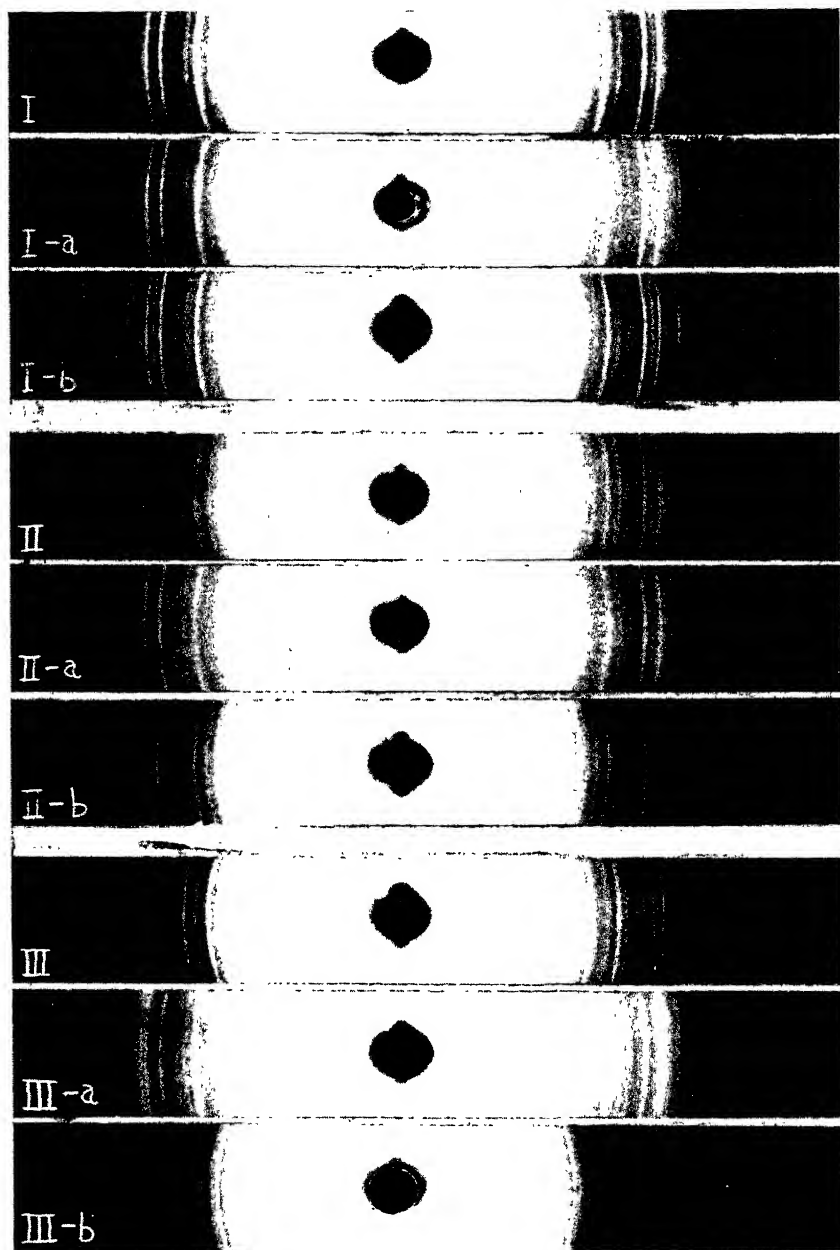
## PLATE 3

Fig. 3. X-ray diffraction patterns of hydroxyapatite (*C*; *C-1* ashed at 900°) and  $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$  (*D*; *D-1*, ashed at 600°; *D-2*, ashed at 900°).





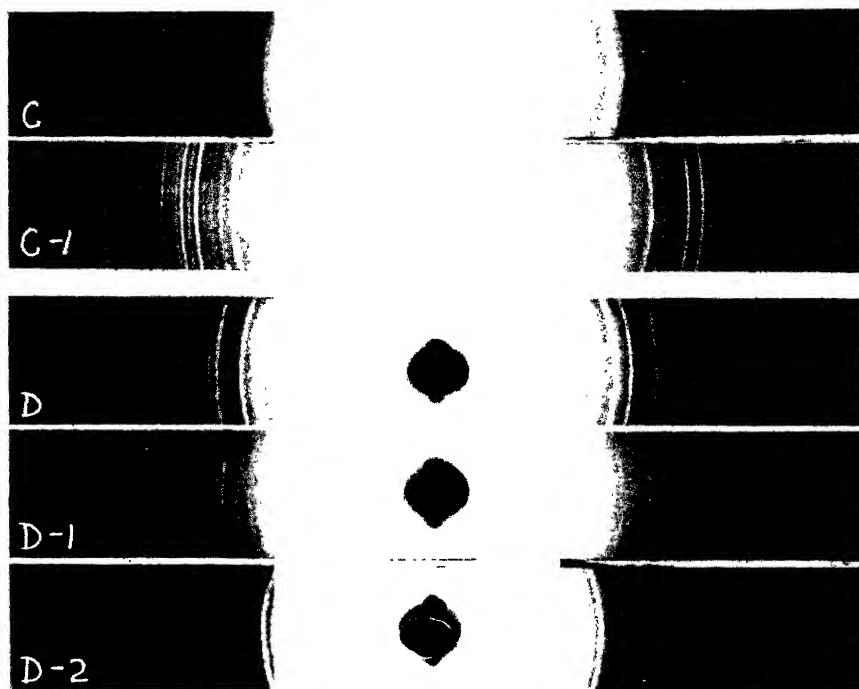




(Hirschman, Sobel, Kramer, and Fankuchen: High phosphate bones)









# ON THE MECHANISM OF ENZYMATIC CONVERSION OF GLUCOSE-1-PHOSPHATE TO GLUCOSE-6- PHOSPHATE\*

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If the formation of an enzyme-substrate complex in the conversion of glucose-1-phosphate (Gl-1-Ph) to glucose-6-phosphate (Gl-6-Ph) under the influence of phosphoglucomutase is granted, then the reaction may be envisioned to occur via one of the following mechanisms:

- (a)  $\text{Gl-1-Ph} + \text{enzyme} \rightleftharpoons \text{enzyme} \cdot \text{Gl} + \text{Ph} \rightleftharpoons \text{Gl-6-Ph} + \text{enzyme}$
- (b)  $\text{Gl-1-Ph} + \text{enzyme} \rightleftharpoons \text{enzyme} \cdot \text{Ph} + \text{Gl} \rightleftharpoons \text{Gl-6-Ph} + \text{enzyme}$
- (c)  $\text{Gl-1-Ph} + \text{enzyme} \rightleftharpoons \text{enzyme} \cdot \text{Gl-1-Ph} \rightleftharpoons \text{enzyme} \cdot \text{Gl-6-Ph} \rightleftharpoons \text{Gl-6-Ph} + \text{enzyme}$

Mechanism (a) depicts the function of phosphoglucomutase to be that of a transglucosidase in that an enzyme-glucose intermediate is formed and there is a subsequent transfer of the glucose to a suitable acceptor (phosphate). That this mechanism, as represented, is not operative in the case of this enzyme was demonstrated by Meyerhof *et al.* (1) with the aid of radioactive phosphorus. These workers demonstrated the failure of radioactive inorganic phosphate, added to the reaction medium, to be incorporated in the Gl-6-Ph formed by the reaction. These findings are incompatible with postulated mechanism (a).

The present investigation was designed to establish which of the remaining postulates, (b) and (c), correctly describes the reaction mechanism. Mechanism (b) postulates the rôle of the enzyme to be that of a transphosphorylase involving labilization of the glucose fragment. Hence, in the presence of radioactive glucose the formation of radioactive Gl-6-Ph should result if the mechanism is valid. The conversion of Gl-1-Ph to Gl-6-Ph was therefore carried out under the influence of phosphoglucomutase in a medium containing radioactive glucose ( $\text{C}^{14}$ ). Under the conditions of the experiment no incorporation of radioactive glucose into the Gl-6-Ph could be detected. From a consideration of these results, taken together with those of Meyerhof *et al.* (1), the conclusion that scheme (c) best represents the reaction mechanism appears to be justified. The results of the present study are in accord with the suppositions implicit in an acceptance of mechanism (c); namely, that the enzyme-substrate intermediate involves the entire glucose phosphate molecule, and further that the ensuing phosphate transfer is strictly intramolecular.

\* Aided by a grant from the John and Mary R. Markle Foundation.

## EXPERIMENTAL

For the conversion of Gl-1-Ph to Gl-6-Ph, 1 ml. of a solution containing potassium glucose-1-phosphate ( $1.39 \times 10^{-2}$  M),  $\text{MnCl}_2$  ( $1.25 \times 10^{-3}$  M), KCN ( $3.3 \times 10^{-3}$  M), radioactive glucose<sup>1</sup> ( $2.78 \times 10^{-2}$  M), and phosphoglucumutase<sup>2</sup> (0.05 mg.) was incubated for 1.5 hours<sup>3</sup> at 37°. A tube containing all of the above materials except for the enzyme served as the control. The pH of both the control and test media was 7.3. Following the incubation period, 1.5 times the calculated amount of barium acetate was added, and the barium hexose phosphate precipitated by the further addition of 1.2 volumes of 95 per cent ethanol. The precipitate was centrifuged, dissolved in 1.0 ml. of  $\text{H}_2\text{O}$ , and reprecipitated with ethanol (1.2 volumes). This procedure of precipitation, solution, and reprecipitation, designed to remove radioactive glucose, was repeated for a total of ten times, the volume concentration of ethanol being gradually increased to 95 per cent.

TABLE I  
*Analysis of Barium Glucose Phosphate for Radioactive Glucose*

	Isolated barium glucose phosphate*	Radioactivity of isolated barium glucose phosphate	Incorporation of glucose ( $\text{C}^{14}$ ) in barium glucose phosphate (corrected for control)
	mg.	counts per min.	per cent
Control.....	2.8	$1 \pm 1$	
Test.....	2.1	$1 \pm 1$	0

\* The barium salt of the control run is Ba Gl-1-Ph. The barium salt of the test run is Ba Gl-6-Ph (97 per cent), Ba Gl-1-Ph (3 per cent).

Finally an ethanol suspension of the precipitated barium glucose phosphate was transferred to an aluminum disk and the radioactivity of the residue determined after evaporation of the alcohol.

## RESULTS AND DISCUSSION

The results of this experiment are summarized in Table I.

It may be seen from the data in Table I that the isolated glucose phosphate (both Gl-1-Ph and Gl-6-Ph) are devoid of radioactivity. From the value of the activity of the original glucose (388 counts per mg. per minute) it is calculated that the presence of as little as 1.0 per cent glucose ( $\text{C}^{14}$ ) in

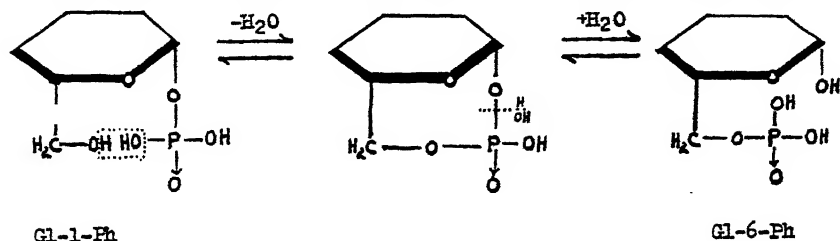
<sup>1</sup> The authors are indebted to Dr. H. A. Barker of the Department of Soil Science for a gift of the  $\text{C}^{14}$ -labeled glucose used in these studies.

<sup>2</sup> The preparation of the phosphoglucumutase free from phosphorylase and hexose isomerase will be described in a separate publication.

<sup>3</sup> Preliminary experiments with non-radioactive glucose revealed that 97 per cent conversion of Gl-1-Ph to Gl-6-Ph is effected in 1.5 hours.

the isolated barium Gl-6-Ph would have been detectable (3.7 counts per minute).

This failure of radioactive glucose in the reaction medium to be incorporated in the Gl-6-Ph (and Gl-1-Ph), as shown in Table I, points to mechanism (c) as best representing the course of the enzymatic conversion of Gl-1-Ph to Gl-6-Ph. In view of the intramolecular nature of the reaction implied by this mechanism, it is of interest to speculate as to the precise mode of transfer of the phosphate group within the glucose molecule from the 1 to the 6 position. A not unreasonable pathway is the formation of an intermediate phosphate diester, as shown in the accompanying diagram.



The plausibility of the idea that this phosphate diester may serve as the intermediate compound is at present best supported by the fact that construction of the diester from Gl-1-Ph or Gl-6-Ph with molecular models reveals that no distortions of bond angles or distances are required for its formation. Further, in the models of Gl-1-Ph or Gl-6-Ph the spatial arrangement of the crucial hydrogen and hydrogen and hydroxyl groups is such that the bond angles and distances characteristic of  $\text{H}_2\text{O}$  are realized. Additional support for the formulation of an intramolecular phosphate diester as an intermediate in the phosphoglucomutase reaction is given by the fact that a similar mechanism has been proposed (2) and proved experimentally (3) for the reaction,  $\beta$ -glycerophosphate  $\rightarrow$   $\alpha$ -glycerophosphate.

#### SUMMARY

Evidence has been presented elucidating the nature of the enzyme-substrate complex involved in the phosphoglucomutase reaction.

A cyclic phosphate diester has been proposed as the intermediate compound in the reversible intramolecular conversion of glucose-1-phosphate to glucose-6-phosphate.

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# THE NATURE OF SOME OF THE CHEMICAL DIFFERENCES AMONG STRAINS OF TOBACCO MOSAIC VIRUS\*

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## PLATE 4

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Between the ordinary protein molecules of the chemist and recognized living organisms there lies a diversified group of obligate parasites which possess some of the properties of both (1); these entities are called viruses. The capacity for self-duplication is a fundamental property of viruses of all descriptions. Closely associated, if not inseparably linked with this property, is the ability of most, if not all, viruses to mutate spontaneously, with the consequent production of physically similar entities which possess, however, altered biological characteristics. These new forms, under proper circumstances, reproduce themselves faithfully and are recognized and designated as strains.

The chief interest in chemical studies on virus strains, if we disregard temporarily the implications which the findings may have in other fields, such as medicine and genetics (2, 3), lies in the light which such investigations may throw upon the currently obscure mechanism of virus multiplication. Furthermore, it might be expected that such studies, if sufficiently extended, would produce results having a bearing on the relationship between chemical structure and biological specificity.

Many strains of tobacco mosaic virus constitute particularly favorable material for analysis, owing to their relatively simple chemical nature and ease of purification. These facts have been utilized in the quantitative determination of nucleic acid and of certain amino acids in strains of tobacco mosaic virus (4-6). The strains analyzed were chosen for their distinctive biological properties and ranged from a masked strain, which shows no readily apparent symptoms in diseased Turkish tobacco plants, to a lethal strain which kills young tobacco plants with regularity. Furthermore, some of the strains were known to be closely related to common tobacco mosaic virus, whereas others were almost surely only distantly related. Qualitative tests showed that all of the strains contain pentosenucleic acid, and the phosphorus analyses indicated that all contain the same proportion of this component. On the other hand, striking differences in protein com-

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position were found in some instances, despite the fact that analyses were made for only a few amino acids. This result suggested that the formation of a virus mutant involves fundamental changes in composition of the virus protein rather than simpler postulated changes, such as a gain or loss of certain reactive groups or a rearrangement of the existing units to form a slightly different pattern. The general nature of the findings made it seem desirable to extend the investigations to include as many amino acids as possible in order to secure a more complete picture of the number and scope of differences among the strains. For this purpose, microbiological methods of assay were employed and, as described herein, these methods revealed many new differences in the composition of the different strains. In addition, these methods made it possible to account for all, or nearly all, of the protein moiety of six of the eight strains analyzed.

### *Materials and Methods*

*Virus Strains. Ordinary Tobacco Mosaic Virus (TMV)*—This is the common green-mottling strain. In Turkish tobacco, it produces a typical mottling, a slight distortion of the leaves, and stunts the growth of the plant (7). Even very young plants, however, are not killed by the virus but grow to maturity, flower, and produce viable seed (7).

*Holmes' Masked Strain (M)*—This strain was obtained by growing at an elevated temperature pieces of tomato stem which had been inoculated with the ordinary strain (8). It is noted for its completely masked character in Turkish tobacco plants in which it is generally not possible to distinguish, by direct observation, infected plants from non-infected, healthy plants of the same age.

*J14D1 Strain*—This strain was obtained by the spontaneous mutation of the J14 strain (9). The J14 strain had in turn arisen by the mutation of ordinary tobacco mosaic virus; that is, it was obtained from a small spot arising on the leaf of a plant infected with the common strain (9). The J14 strain is characterized by its production of necrotic lesions on the inoculated leaves of Turkish tobacco, and by its non-systemic nature and low infectivity (7, 9). On the other hand, the J14D1 strain produces necrotic primary lesions and then becomes systemic, producing necrotic spots and also a characteristic yellowing of the upper leaves of the infected plants. It is much more infectious than the J14 strain from which it was derived. In striking contrast to most of the strains of tobacco mosaic virus, the J14D1 strain is lethal to young Turkish tobacco plants. It also kills tomato plants with great regularity, including large plants at the flowering stage (7). The sequence of events following infection of young Turkish tobacco with J14D1 is illustrated in Text-Fig. 1.

*Yellow Aucuba Strain (YA)*—This is the aucuba mosaic of tomato first

reported by Bewley and studied by a number of other workers (10). In Turkish tobacco, it produces a systemic disease marked by a pronounced yellowing of the foliage.

*Green Aucuba Strain (GA)*—This was obtained by growing the yellow aucuba strain in a species of tobacco at an elevated temperature (10). The new strain cannot be distinguished from yellow aucuba in many hosts, but differs from it in Turkish tobacco in which its symptoms are virtually indistinguishable from those of ordinary tobacco mosaic virus. It can be differentiated from the latter by inoculation to *Nicotiana sylvestris* in which, unlike ordinary tobacco mosaic virus, it produces necrotic primary lesions.

*Holmes' Rib-Grass Strain (HR)*—This strain, originally isolated from rib-grass, produces distinctive necrotic ring patterns in Turkish tobacco (11).



TEXT-FIG. 1. Stages in the progress of the disease caused by the J14D1 strain of tobacco mosaic virus in Turkish tobacco. In the first plant, on the left, can be seen necrotic spots marking the primary sites of infection; in the second plant, extensive chlorosis of several upper leaves marks the systemic spread of the virus; on the right is demonstrated the effect of the final stage of the disease which has resulted in the death of the plant. Photograph by J. A. Carlile

*Cucumber Viruses 3 and 4 (CV3 and CV4)*—These two viruses grow only in plants of the Cucurbitaceae in which strain CV3 produces a green mottling and CV4 a brilliant yellow mottling. While these are presumed to be strains of tobacco mosaic virus, the proof for this is less complete than it is for the strains described above (12).

Illustrations of some of the symptoms produced in Turkish tobacco by the strains just described are given in a previous report (4) and will supplement the data given above.

*Preparation of Virus for Analysis*—Highly purified preparations of virus were obtained from groups of appropriately diseased plants by the procedures previously described (12). Many of the samples used were those on which chemical analyses had also been made (4-6). All preparations were dried in a manner previously described (4) and hydrolysates of the

viruses were obtained by heating 40 to 60 mg. samples in 2 ml. portions of 2.7 N hydrochloric acid in sealed tubes in an autoclave at 15 pounds pressure (121°) for 10 to 12 hours. The hydrolysates were neutralized with 10 per cent sodium hydroxide, filtered, and the combined filtrate and washings for each sample were brought to a volume of 250 ml. For the tryptophan assays, separate samples of 10 to 15 mg. were hydrolyzed in 1 ml. portions of 20 per cent sodium hydroxide in sealed tubes in an autoclave at 15 pounds pressure for 15 hours. The hydrolysates were neutralized, filtered, and brought to a volume of 100 ml.

*Microbiological Assays and Other Analyses*—The methods used were largely those of Stokes and coworkers (13–15), to whom the author is also indebted for original cultures of the bacteria employed. No methods were described by Stokes and collaborators for the determination of alanine, glutamic acid, proline, and glycine. However, these amino acids were determined in the present investigation by methods which satisfied fairly well the usual criteria of reliability in microbiological assays (13). Other additions to or deviations from the Stokes procedures included the use of *Leuconostoc mesenteroides* P-60 (16), as well as *Streptococcus faecalis*, to determine lysine, and the use of *L. mesenteroides* in the aspartic acid assays rather than *Lactobacillus delbrueckii*. Thus, *Streptococcus faecalis* was employed in assays for alanine, arginine, glutamic acid, histidine, isoleucine, leucine, lysine, methionine, threonine, tryptophan, and valine. *L. delbrueckii* LD5 was used to determine phenylalanine, serine, and tyrosine, and *L. mesenteroides* was employed in assays for proline, aspartic acid, glycine, and lysine.

Cysteine was determined in solutions of virus by a colorimetric procedure involving the reduction of Folin's uric acid reagent in the presence of urea (17). The absence of cystine was previously established for several strains by chemical means (6, 12, 18) and can be assumed for some of the others by virtue of the fact that the sulfur present can all be accounted for as cysteine. In the case of J14D1 strain, additional tests were made which showed that the color obtained with nitroprusside was not enhanced by addition of sodium cyanide (19) and hence that cystine was absent.

The results of the analyses are presented in Table I, together with the mean deviation of the values of single determinations from the averages given. The latter calculation was not made in the case of the cysteine values because there was an insufficient number of analyses in some instances. However, no significance is attached to the variations in cysteine values listed in Table I. In most of the microbiological assays, three to five preparations of each strain were assayed at three different levels. At least two preparations were analyzed in every instance. The lysine value for J14D1 obtained with *Streptococcus faecalis* was checked by assay of two preparations with *Leuconostoc mesenteroides*. The values obtained were

1.98 and 1.95 per cent, which agree very well with the results obtained with *Streptococcus faecalis*.

*Chromatography*—Contrary to the results of earlier chemical studies (21),

TABLE I  
Amino Acid Content of Highly Purified Preparations of Some Strains of Tobacco Mosaic Virus\*

Amino acid	Strain								
	TMV	M	J14D1	GA	YA	HR	CV3	CV4	M.D.†
Alanine.....	5.1	5.2	4.8	5.1	5.1	6.4		6.1	0.2
Arginine.....	9.8	9.9	10.0	11.1	11.2	9.9	9.3	9.3	0.2
Aspartic acid.....	13.5	13.5	13.4	13.7	13.8	12.6		13.1	0.2
Cysteine.....	0.69	0.67	0.64	0.60	0.60	0.70	0	0	
Cystine.....	0		0			0		0	
Glutamic acid.....	11.3	11.5	10.4	11.5	11.3	15.5	6.4	6.5	0.2
Glycine.....	1.9	1.7	1.9	1.9	1.8	1.3	1.2	1.5	0.1
Histidine.....	0	0	0	0	0	0.72	0	0	0.01
Isoleucine‡.....	6.6	6.7	6.6	5.7	5.7	5.9	5.4	4.6	0.2
Leucine.....	9.3	9.3	9.4	9.2	9.4	9.0	9.3	9.4	0.2
Lysine.....	1.47	1.49	1.95	1.45	1.47	1.51	2.55	2.43	0.04
Methionine.....	0	0	0	0	0	2.2	0	0	0.1
Phenylalanine.....	8.4	8.4	8.4	8.3	8.4	5.4	9.9	9.8	0.2
Proline.....	5.8	5.9	5.5	5.8	5.7	5.5		5.7	0.2
Serine.....	7.2	7.0	6.8	7.0	7.1	5.7	9.3	9.4	0.3
Threonine.....	9.9	10.1	10.0	10.4	10.1	8.2	6.9	7.0	0.1
Tryptophan.....	2.1	2.2	2.2	2.1	2.1	1.4	0.5	0.5	0.1
Tyrosine.....	3.8	3.8	3.9	3.7	3.7	6.8	3.8	3.7	0.1
Valine.....	9.2	9.0	8.9	8.8	9.1	6.2	8.8	8.9	0.2

\* The values given in the table represent percentages of the indicated amino acids. In order to facilitate comparison, the values which are considered to differ significantly from those of TMV are in bold-faced type.

† Mean deviation of the values of single determinations from the averages given. Three to five preparations of each strain were analyzed for each amino acid, with the exception of cysteine, and the results were averaged to give the figures presented.

‡ Towards the end of the present investigation, a purer isoleucine standard became available. Assays of TMV in which this standard was employed yielded an average value of 6.6 per cent of isoleucine instead of 8.4 per cent yielded by the earlier standard. The figures for the other strains which were obtained with the earlier standard have been corrected proportionately, as should also be the isoleucine values given in (20).

the microbiological assays indicated that TMV contains glycine. The other strains examined also appeared to contain this amino acid. While there was little reason to suspect the results of the microbiological assays, nevertheless it seemed desirable, in view of the conflict with the chemical

data, to seek other evidence which would either confirm or deny the presence of glycine. The paper chromatography method of Consden and coworkers (22) was selected for this purpose.

600 mg. of TMV were hydrolyzed in 10 ml. of 2.7 N hydrochloric acid in a sealed tube in an autoclave at 15 pounds pressure for 11 hours. The hydrolysate was transferred with washings to an electro dialysis apparatus in which the removal of most of the hydrochloric acid and the separation of the neutral from the basic amino acids and part of the acidic amino acids was effected. The mixture of amino acids remaining in the central chamber of the electro dialysis apparatus was concentrated *in vacuo* to about 1 ml. Tiny drops of this concentrate were applied to the strips of filter paper with a finely drawn capillary tube. One-dimensional chromatograms were made with phenol in the presence of a trace of hydrogen cyanide (22). The paper employed was Munktell quick filtering paper, No. OB.

Three of the chromatograms obtained are reproduced in Fig. 1. These demonstrate beyond reasonable doubt the presence of glycine in hydrolysates of TMV.

#### DISCUSSION

The microbiological assay methods employed in the present investigation are considered to possess an accuracy of  $\pm 10$  per cent (23). However, as judged by the values given in Table I for the mean deviation of single analyses from averages, it is apparent that the reproducibility of the individual assay values was, in most cases, considerably better than this. For example, the arginine values for strains GA and YA were only about 10 per cent higher than those for TMV and some of the other strains, but the reproducibility of this difference was such as to leave little doubt of its validity. If needed, however, confirmation of the existence of this particular difference is available in the chemical analyses previously made (5). Thus it is believed that the mean deviations presented in Table I provide a good estimate of the reproducibility of each analysis and hence a basis for a judgment of which of the observed differences are probably significant.

A conversion of the amino acid percentages into percentages of amino acid residues, that is, amino acid less the molecule of water lost in the formation of a peptide bond, followed by summation of the results, leads to totals which, when added to the approximately 6 per cent nucleic acid of the virus, account for about 98 per cent of the various strains, with the exceptions of strains CV3 and CV4. The total for strain CV4 was about 90 per cent. Similarly, when the nitrogen contents of the amino acids of each strain were totaled and added to the nitrogen of the nucleic acid and the 1.5 per cent amide nitrogen presumed to be present (24), the sums ranged from about 99 to 102 per cent of the total nitrogen of the strains found by analysis.

Again the cucumber viruses were exceptions, the CV3 strain owing partly to incomplete analysis and the CV4 strain owing presumably to the presence of amino acids other than those for which analyses were made or to the presence of unnatural isomers. Other evidence suggests the presence of uncommon amino acids in strain CV4 (12). In general, the two summations just described suggest that the present analyses account for all or nearly all of the protein of each of the strains with the exception of strains CV3 and CV4,<sup>1</sup> but this point will be more firmly established if repeated analyses, perhaps with other organisms or other methods, should yield essentially the same results. In the meantime, however, this assumption provides a useful basis for speculation and further experimentation.

In the cases of most of the strains employed in this study, many amino acids were determined for the first time and hence the results cannot be compared with those obtained previously. Values had been obtained for most of the common amino acids in the case of TMV (24), but these earlier figures are, with four or five exceptions, considerably lower than those found in the present assays. This is readily explained by the fact that many of the earlier results, which have furnished an excellent foundation for subsequent work, were procured by means of isolation methods which admittedly involved considerable losses.

The values obtained by Stokes and collaborators in the microbiological assay of a single preparation of TMV for eleven amino acids (14) agree within about 10 per cent in eight instances with the average results reported herein for analyses of five different preparations of TMV; however, discrepancies of 20 to 30 per cent are observed in the values for phenylalanine, valine, and leucine, and in all of these instances the present values are higher than those of Stokes. In the case of valine, determinations kindly made by Dr. Stokes on a fresh hydrolysate of TMV provided by the author yielded an average value of 8.3 per cent, which is closer to the figure given in Table I than to the value previously obtained by Stokes on the same preparation (14). The discrepancies in values for phenylalanine and leucine have not yet been resolved, although, as mentioned elsewhere in this discussion, the value for phenylalanine given in Table I agrees well with the results obtained in many analyses made by a colorimetric method.

Comparisons between chemical and microbiological results can be made for all strains in the cases of the aromatic and basic amino acids. The tyrosine values obtained by the two methods agree very well, and the microbiological data confirm the absence of histidine from all except one of the

<sup>1</sup> This argument is strengthened in the case of TMV by the results of preliminary two-dimensional chromatographic analyses. These have failed to reveal the presence in significant amounts of amino acids other than those for which values are recorded in Table I (Knight, C. A., unpublished data).

strains. The histidine value for the exceptional strain and the arginine values obtained by the present assays are somewhat higher than those of the chemical analyses, but the nature of the latter procedures was such that slightly low values would be expected. The suspected presence of a small amount of lysine in the strains (5) was amply confirmed by the present assays. The microbiological tryptophan values proved to be very much lower than those obtained by the glyoxylic acid method (4); on the other hand they agreed very closely, in the case of TMV, with the figures obtained in this and another laboratory by a different chemical procedure (21, 25). It seems probable, therefore, that the actual tryptophan content of tobacco mosaic virus is about 2 per cent rather than 4.5 per cent, although no explanation is yet available for the consistently higher values given by the glyoxylic acid method. It should be noted, in connection with acceptance of the lower tryptophan values for the various strains, that they preserve the same relationships to each other as did the higher values and hence the conclusions drawn from the earlier data are in no way invalidated. Acceptance of the lower tryptophan values, however, does necessitate recalculations of the previous phenylalanine values, since these involved a correction for the tryptophan content of the protein (4). Such calculations yield figures ranging from about 8.7 to 9.0 rather than about 6 per cent phenylalanine for strains TMV, M, J14D1, GA, and YA, about 6.4 rather than 4.3 per cent for strain HR, and about 11 rather than 10 per cent for strains CV3 and CV4. These recalculated results are in good agreement with the microbiological assays in most cases, although in only fair agreement in the remaining instances.

Assays with *Leuconostoc mesenteroides* indicated that all of the strains contain glycine, although it had been concluded from earlier chemical studies that TMV, at least, was devoid of this amino acid (21). Although the glycine assay is new and has not been tested as thoroughly as some of the other assays, there appears to be no good reason for doubting the positive indication of the presence of 1 to 2 per cent of glycine in several strains. Strong support for this conclusion is supplied by the results obtained in the application of paper chromatography to hydrolysates of TMV. This method demonstrated beyond reasonable doubt the presence of glycine in hydrolysates of TMV. It is interesting in this connection that TMV appears to contain a significant amount of streptogenin and that the latter is presumably a peptide containing serine, glutamic acid, and glycine (26, 27).

It was thought, before completion of the present analyses, that the differences in protein composition among strains might be confined to certain amino acids which possess the most reactive chemical groups. However, Table I shows that sixteen of the nineteen amino acids determined were involved in the differences found. It is thus virtually certain that mutation

of these viruses can involve any amino acid contained in the protein and may even involve the addition of amino acids which were not previously present. For example, the HR strain contains histidine and methionine, two amino acids which are absent from the other strains examined. It is necessary to postulate either that the strains without histidine and methionine arose as a result of loss of these amino acids, perhaps preceded or followed by changes in other components, or histidine and methionine were added to a virus molecule which previously contained none. In any case, it appears that mutation of tobacco mosaic virus or of its strains can be accompanied by a change in relative proportion of any of the amino acids.

In addition to confirming all of the previously known differences in composition between the various strains and the typical TMV, the microbiological assays revealed twenty-five new ones. Most of these were found in strains HR, CV3, and CV4, for the results of Table I show only two significant differences between TMV and each of the strains GA, YA, and J14D1. No chemical distinction between TMV and M has as yet been found. In general the results strongly suggest that there is a definite correlation between the number of demonstrable chemical differences and the degree of relationship of the virus strains. For example, the HR strain, which was found to differ chemically in many respects from TMV, is almost certainly a distant relative of the latter in view of the fact that variants of its type have never been observed from mutations of TMV, although dozens which are representative of other types are readily procured (9). Similarly, mutants of strain HR are occasionally observed, but these have always resembled strain HR rather than TMV or any of the mutants derived therefrom.<sup>2</sup> On the other hand, the J14D1 strain, which was obtained from TMV, presumably through two successive mutations, was found to differ from TMV with respect to only two amino acids. Likewise, the GA and YA strains were found to differ from TMV in only two respects. While the precise relationship of strains YA and GA to TMV is not known, it is undoubtedly close, for closely similar strains are frequently obtained as spontaneous mutants of TMV (9). Additional evidence in support of the view-point that distantly related strains of a virus possess numerous chemical differences is available in the one instance in which two presumably distantly related strains of a virus affecting animals were compared (20).

The findings with the J14D1 strain have a bearing on the question of how much change in chemical composition accompanies a single mutation. Since this strain is believed to have arisen from TMV by two mutations and since two differences in composition were found, it would seem reasonable to conclude that each mutation involved an analytically significant

<sup>2</sup> Holmes, F. O., personal communication.



change in proportion of one amino acid. This hypothesis could be tested by isolation and analysis of the first mutant as well as the second. However, the first mutant in this case (J14 strain) has been lost, and it will be necessary to isolate a new series of successive mutants in order to make such a test.

A comparison of the composition of TMV and of J14D1 shows in a striking manner how small the chemical differences need be between a virus which kills a given host and one which does not. If these results were translated to the field of animal viruses, it would be easy to understand the sudden conversion of a relatively mild epidemic virus to a lethal form. Such an event may possibly have occurred in the formation of the virus strain responsible for the influenza pandemic of 1918-19.

No chemical differences between TMV and M or between strains GA and YA were revealed in the present study. The M strain is known to be very closely related to the TMV strain (8), and in Turkish tobacco it frequently mutates to give TMV or closely allied strains. Its isoelectric point<sup>3</sup> and general physical and chemical properties are indistinguishable from those of TMV and evidently the chemical difference between them must be slight. The distinction may reside in a difference in amino acid content too small to be detected by the present assays; or an amino acid may be involved which was not determined in this investigation. An alternative hypothesis is that the change involves the nucleic acid in some obscure manner. In the case of the pair of strains YA and GA, an analogous situation exists, and indeed, the GA strain was procured from strain YA in the same manner that strain M was obtained from TMV. The YA and GA strains are so closely related that they produce the same symptoms in many hosts, but differ in Turkish tobacco. Even in Turkish tobacco, however, the symptoms produced by the two strains are indistinguishable during the short days of winter (28).

The type of chemical difference observed thus far between virus strains is of a sort which would probably not be obtained by the direct action of chemical reagents or radiations on the virus particles themselves. That is, an incorporation into or removal from the peptide structure of the viral protein of a thousand or more amino acid residues of a particular kind is a type of change which one would not expect to effect directly by chemical action or by radiations. This may explain the failure, as yet, of attempts to produce unequivocal heritable changes by these means (29-32). However, this approach has not been pursued exhaustively and may eventually succeed. In the case of the chemical derivatives of TMV, for example, it is possible that the proper type of substituent has not yet been selected. Also, if radiations are capable of producing mutations in viruses, the

<sup>3</sup> Oster, G., to be published.

number of mutants would be expected to be low, and present methods of testing for mutants among strains of tobacco mosaic virus are such that it would be difficult, if not impossible, to distinguish small numbers of induced mutants from the few spontaneous ones which are found in virtually every preparation of virus. Finally, it can be postulated that small but vital changes in the virus might be initiated by chemical reagents or radiations and that these would, in the subsequent multiplication of the virus particles, result in secondary more profound alterations in the protein composition. The results of the amino acid analyses in two instances discussed above contain a hint that there may be more than one type of chemical change associated with virus mutation. In this connection it should be noted that, while there is at present little direct chemical evidence for the existence of differences among the nucleic acid components of strains of tobacco mosaic virus, this possibility has not been thoroughly investigated.

It is a pleasure to acknowledge the counsel of Dr. W. M. Stanley, who initiated studies on the composition of strains of tobacco mosaic virus some years ago, and whose sustained interest has facilitated subsequent progress in the investigation along these lines.

#### SUMMARY

Chemical and microbiological assays for nineteen amino acids were made on highly purified preparations of eight strains of tobacco mosaic virus. One strain was found to contain histidine and methionine, amino acids which are entirely absent in the other seven strains. In addition, thirty-eight quantitative differences in one amino acid or another were found among the strains. In general, those strains which, from biological properties, appeared to be most distantly related to the type strain showed the most marked differences in protein composition. One strain, which is believed to have arisen from the type strain by two mutations or variations, was found to differ in composition from the type strain in only two respects. The suggestion is made that, in some cases at least, mutation among tobacco mosaic viruses involves stepwise changes in amino acid content.

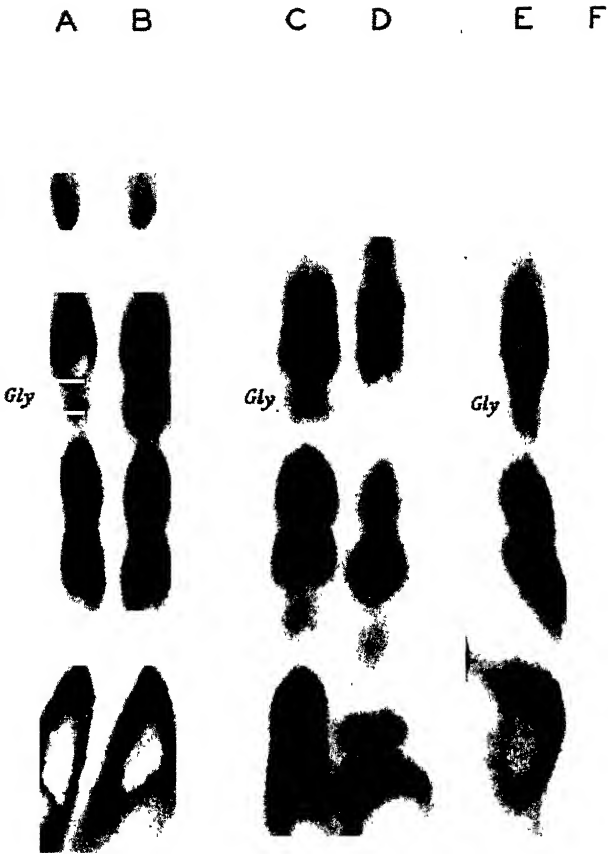
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## EXPLANATION OF PLATE 4

FIG. 1. One-dimensional chromatograms of an electrodyalyzed hydrolysate of tobacco mosaic virus (TMV) and of some amino acids run in phenol for about 8 hours on Munktell quick filtering paper, No. OB. The spots were developed by spraying the paper with ninhydrin solution and heating. A, TMV hydrolysate; B, TMV hydrolysate plus glycine (note intensification of glycine spot); C, TMV hydrolysate; D, mixture of all amino acids known to be present in TMV except glycine (note the absence of a spot in the glycine area); E, TMV hydrolysate; F, glycine, 1 mg. per ml. (Photograph by J. A. Carlile.)



(Knight: Tobacco mosaic virus)



# DISTRIBUTION OF A LARGE DOSE OF THYROXINE LABELED WITH RADIOIODINE IN THE ORGANS AND TISSUES OF THE RAT\*

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In the past the distribution of thyroid hormone in the peripheral tissues has been determined by chemical and biological methods which were often rather crude and non-specific. The chief consistent finding of these early investigations was that thyroid hormone disappears rapidly from the blood and is fixed in large amounts in the liver (1, 2). The results obtained by various authors in other organs are somewhat contradictory and will be discussed below. It was therefore considered of interest to reexamine the problem with the use of thyroxine labeled with radioactive iodine,  $I^{131}$ . This method of approach was first applied in the rabbit (3-5). Few biological results were obtained, the most important being the finding of radiothyroxine in the liver and hypophysis 5 hours after intravenous injection (5).

## Methods

Radioactive iodine ( $I^{131}$ ) was obtained either as iodide from the cyclotron of the Massachusetts Institute of Technology or as a deuteron-bombarded tellurium target from the cyclotron of Washington University. In the latter case, radioiodine was prepared in the following manner: The tellurium metal was placed in a 500 cc. round bottom boiling flask connected to a ground joint incorporating a dropping funnel and fused to a U-shaped glass tube. The other end of the tube dipped into 2 cc. of 60 per cent chromic acid so as to trap any fumes evolved in the flask. 25 cc. of concentrated sulfuric acid were added to the tellurium through the dropping funnel, the flask then being vigorously heated. The tellurium dissolved with a red color. On being boiled further, the solution became colorless and a fine white precipitate settled out. The chromic acid (which trapped a not inconsiderable portion of radioactivity) was washed into the flask, which was then connected to a Chaney apparatus and distilled according to this author's method (6). The radioactive iodine was received in sodium hydroxide and was therefore available as iodide.

\* Preliminary reports of this work were presented by C. P. Leblond before the New York Academy of Sciences on January 25, 1946, and by J. Gross before the American Association of Anatomists (*Anat. Rec.*, suppl., 97, 339 (1947)).

Radioactive thyroxine was prepared from DL-diiodothyronine<sup>1</sup> by the method of Horeau and S   (3) and injected intravenously into rats as a faintly alkaline solution. To identify the material as thyroxine, it was shown (a) that the Kendall-Osterberg reaction, characteristic of ortho-iodinated phenols, was positive while diiodothyronine does not give this test, (b) that the melting point of the material (229-230 ) was in the same range as that of commercial DL-thyroxine and a mixed melting point showed no depression, the melting point of diiodothyronine being 216-219 , and (c) that the material had a biological activity identical to that of thyroxine, as shown by daily injections of 50   of thyroxine for 6 days into thyroidectomized male rats weighing 130 to 150 gm. (Table I). This

TABLE I

*Effect on Oxygen Consumption of Labeled Thyroxine Preparation and Labeled Thyroxine Recovered from Feces*

The experimental animals received doses of material equivalent in iodine content to 50   of thyroxine in 0.1 cc. of sodium hydroxide solution adjusted to pH 8 to 9. There were six animals in each group.

	Oxygen consumption, cc. per sq.m. body surface per hr.	
	Before injection period	At end of injection period
Controls (receiving NaOH solution only)	60.3 $\pm$ 2.7	55.9 $\pm$ 1.97
Butanol extract of feces from labeled thyroxine-treated animals	65.5 $\pm$ 1.78	81.3 $\pm$ 3.48
Thyroxine prepared from DL-diiodothyronine	65.2 $\pm$ 2.42	97.9 $\pm$ 4.75
Commercial DL-thyroxine (Hoffmann-La Roche)	64.3 $\pm$ 2.28	91.6 $\pm$ 2.72

experiment, which was repeated twice with the same results, clearly showed that thyroxine prepared from DL-diiodothyronine was as active as commercial thyroxine.

Female albino rats weighing from 70 to 100 gm. were used in the experiments on the distribution of labeled thyroxine. They were sacrificed either 2 or 24 hours after injection. All of the blood was removed from the anesthetized animals as completely as possible by aspiration from the inferior vena cava. The results (Table II) refer to four groups of two animals each ((a) and (b)), in which the distribution of radiothyroxine was examined in detail after administration of doses ranging from 0.8 to 7 mg.

After the animals were sacrificed, the organs were removed and weighed. Each organ was then homogenized in 2 N NaOH, 2 cc. being used for each

<sup>1</sup> Kindly supplied by Hoffmann-La Roche, Ltd., Montreal, through the courtesy of Mr. Paul Blanc.

100 mg. of tissue. Organs weighing less than 100 mg. were each placed in 2 cc. of 2 N NaOH. The organs were then heated on a water bath until completely dissolved.

For the measurement of radioactivity on the Geiger counter, 2 cc. of the solution were evaporated to dryness on a 40 mm. watch-glass in an oven at 70°, and then placed below the window of a bell type  $\beta$ -ray counting tube. The blood plasma was calculated as being 2.15 per cent, the muscles 50 per cent, and the lymphatic tissue 0.5 per cent of the body weight. The other organs were estimated *in toto*. The blood cells were treated after having been washed three times with saline. The content of the gastrointestinal tract was expressed from the tubes but no washing of the walls was carried out. The Geiger counter readings, which were within the range of the variations of the counter background, were expressed as less than 3 times the standard error of the background values (Table II). For each animal, an aliquot of the injected material was plated and counted in 2 cc. of 2 N NaOH. This made possible an estimate of the percentage of the injected dose present in the organs. In addition to the percentages of the injected dose per organ, the concentrations of radioactivity were listed in Table II. These were calculated as the ratio of the number of counts per mg. of organ weight over the number of counts injected per mg. of body weight.

Any remaining quantity of organ solutions was hydrolyzed for at least 4 hours more with gentle boiling and then shaken with *n*-butanol according to Blau's directions (7). The butyl alcohol-soluble fraction was evaporated, the residue being taken up in 2 N NaOH and counted. In the case of the smaller organs, the material was washed off the watch-glass used for counting it and hydrolyzed as above. It has been indicated (8, 9) that such methods permit the recovery of 74 to 84 per cent of added thyroxine. In spite of expected low recoveries, the separations were carried out and may be taken to indicate the general trend of the results. The separation results obtained from the animals given 0.8 mg. of thyroxine (Table III) were similar to those found in other groups.

The radioactivity in the combined alkaline washings was identified as iodide by the following method: 50 mg. of inactive potassium iodide were added to the washings, which were then neutralized with concentrated nitric acid and precipitated with a 10 per cent solution of silver nitrate in 2 per cent nitric acid. Under these conditions, iodide, but neither diiodo-tyrosine nor thyroxine, is precipitated. The precipitate was washed with ammonia to remove chlorides and counted. Thus by applying this technique to the alkaline fractions of stomach and urine, it could be shown that the radioactivity existed predominantly as iodide.

In order to analyze the rôle played by the various sections of the gastro-



TABLE II

*Localization of DL-Thyroxine Labeled with  $I^{131}$* 

Concentration = ( $I^{131}$  per mg. organ weight)/( $I^{131}$  per mg. body weight).

(a) and (b) refer to values found in two separate animals for each dose at each time interval, the results on eight separate animals being given in the table. Values preceded by < were used for those organs whose radioactive content was less than 3 times the standard error of the background counting rate.

	2 hrs. after thyroxine injection					24 hrs. after thyroxine injection			
	2 mg.		0.8 mg.			0.8 mg.		7 mg.	
	Per cent of injected dose	Concentration	Per cent of injected dose	Concentration		Per cent of injected dose	Concentration	Per cent of injected dose	Concentration
Blood plasma	(a) 0.72	0.31	1.84	0.85		0.12	0.06	0.05	0.03
	(b) 1.00	0.46	2.23	1.04		0.20	0.09	0.05	0.02
Gastrointestinal tract									
Stomach	(a) 0.70	0.97				0.16	0.19	0.03	0.05
	(b) 0.39	0.55	1.06	1.43		0.07	0.06	<0.03	<0.03
" content	(a) 3.27	3.83	5.30	3.55		0.17	0.09	0.02	0.12
	(b) 0.36	1.48	2.36	4.86		0.13	0.10	<0.03	<0.03
Duodenum	(a) 0.63	1.60	0.24	0.58		0.03	0.08	0.04	0.09
	(b) 0.62	3.80	0.27	1.06		0.04	0.10	0.05	0.09
Duodenal content	(a) 2.34	3.16	0.90	2.69		0.008	0.10	0.009	0.07
	(b) 0.95	2.51	0.34	1.80		0.008	0.12	0.007	0.04
Jejunum	(a) 29.02	9.40	7.52	3.14		0.47	0.19	0.22	0.11
	(b) 5.33	1.89	9.00	3.21		0.34	0.14	0.22	0.07
" content	(a) 21.21	23.80	38.60	14.81		0.16	0.08	0.31	0.30
	(b) 24.11	12.50	36.65	27.40		0.20	0.14	0.20	0.06
Colon-ecum	(a) 1.33	0.76	0.21	0.14		0.12	0.09	0.25	0.21
	(b) 1.98	0.71	0.43	0.40		0.12	0.10	0.10	0.07
Colon-cecal content	(a) 12.34	4.40	0.33	0.12		0.25	0.24	8.16	4.50
	(b) 3.69	1.95	0.70	0.37		0.97	0.56	1.75	0.55
Feces	(a)		0.12			66.50		86.40	
	(b)					70.50		94.90	
Glands related to gastrointestinal tract									
Liver	(a) 13.81	3.06	9.85	2.16		8.30	1.85	4.39	0.99
	(b) 9.85	2.28	24.30	3.40		8.05	1.98	3.90	0.60
Pancreas	(a) 0.26	0.50	0.19	0.41		0.03	0.08	0.02	0.04
	(b) 0.39	0.91	0.19	0.54		0.03	0.06	0.02	0.03
Submaxillary	(a) 0.05	0.25	0.06	0.28		0.006	0.03	0.005	0.03
	(b) 0.04	0.27	0.11	0.52		0.007	0.04	0.004	0.02
Sublingual	(a)		0.01	0.28		<0.002	<0.05	<0.004	<0.07
	(b) 0.02	0.30	0.02	0.46		<0.003	<0.07	<0.003	<0.05
Parotid	(a)		0.02	0.32		<0.002	<0.07	<0.004	<0.06
	(b) <0.02	<0.43				0.003	0.03	0.003	0.03

TABLE II—*Concluded*

	2 hrs. after thyroxine injection				24 hrs. after thyroxine injection			
	2 mg.		0.8 mg.		0.8 mg.		7 mg.	
	Per cent of injected dose	Concentration	Per cent of injected dose	Concentration	Per cent of injected dose	Concentration	Per cent of injected dose	Concentration
Urinary system								
Kidneys	(a) 0.61	0.66	0.95	1.09	0.31	0.34	0.26	0.25
	(b) 1.43	1.51	2.49	2.00	0.27	0.32	0.23	0.19
Urine	(a)		0.80		13.10		3.13	
	(b)		6.10		26.30		3.44	
Endocrine glands								
Thyroids	(a) 0.12	40.30	0.09	1.21	0.25	28.86	0.03	3.38
	(b) 9.15	11.20	0.06	5.10	0.25	22.50	0.06	3.13
Adrenals	(a) <0.02	<0.49	0.01	0.35	0.010	0.30	0.010	0.28
	(b) <0.02	<0.51	0.04	0.80	0.006	0.30	0.007	0.15
Ovaries	(a) 0.080*	1.90	0.02*	0.40	0.002	0.13	0.01*	0.31
	(b) <0.02	<0.13	0.005	0.42	0.004	0.21	0.01	0.13
Pituitary	(a) <0.02	<2.74	<0.002	<0.54	<0.002	<0.45	<0.004	<0.55
	(b) <0.02	<4.39	<0.002	<0.52	<0.003	<0.56	<0.003	<0.49
Lymphatic system								
Spleen	(a) 0.50	1.16	0.51	0.60	0.72	0.96	0.28	0.27
	(b) 0.69	1.34	1.75	0.59	0.50	0.62	0.27	0.20
Lymph nodes	(a) 0.16	0.14			0.09	0.19	0.03	0.07
	(b) 0.15	0.29	0.28	0.57	0.18	0.36	0.04	0.08
Thymus	(a) 0.02	0.10	0.03	0.12	0.06	0.18	0.004	0.02
	(b) 0.04	0.10	0.04	0.27	0.007	0.02	<0.003	<0.009
Lungs	(a) 2.00	2.82	1.72	2.58	0.41	0.39	0.13	0.18
	(b) 0.70	1.15	1.41	0.97	0.28	0.35	0.04	0.05
Skin	(a) 1.97	0.19	5.94	0.41	1.15	0.09	0.63	0.04
	(b) 3.54	0.32	4.55	0.38	1.31	0.08	0.51	0.02
Skeletal muscle	(a) 6.60	0.14	7.80	0.16	1.14	0.02	0.81	0.02
	(b) 13.30	0.26	18.50	0.38	1.33	0.03	<1.59	<0.03
Cardiac muscle	(a) 0.07	0.24	0.21	0.50	0.02	0.06	0.02	0.04
	(b) 0.10	0.30	0.20	0.44	0.02	0.06	0.03	0.04
Uterus	(a) 0.03	0.13	0.05	0.24	0.002	0.04	0.007	0.05
	(b) 0.02	0.24	0.04	0.78	0.004	0.08	0.004	0.03
Background	20	20	29	26	27	25	14	17
	±3.2	±3.2	±2.6	±2.84	±2.8	±2.9	±3.8	±3.4

\* Corpora lutea present.

intestinal tract in secreting the large amounts of thyroxine found in the intestinal lumen, two groups of adult male rats were used, one group consisting of intact control animals, the other being treated as follows: 2

TABLE III

*Separation of Thyroxine in Organs of Animals Treated with 0.8 Mg. of Labeled Thyroxine*

	Per cent of radioactivity recovered in butyl fraction		Calculation of per cent of injected dose recovered as thyroxine	
	2 hrs	24 hrs.	2 hrs.	24 hrs.
Plasma	25	54	0.51	0.09
Gastrointestinal tract				
Stomach content	8	30	0.31	0.04
Jejunioileal content	72	69	6.63	0.25
Colon-cecal "	52	81	0.28	0.49
Feces		52		35.40
Glands related to gastrointestinal tract				
Liver	77	63	13.12	5.15
Pancreas	42	50	0.08	0.02
Urinary system				
Kidney	64	48	1.10	0.14
Urine	2	15	0.01	2.86
Skeletal muscle	93		12.55	

TABLE IV

*Distribution of Large Dose of Thyroxine (1.4 Mg.) after Separation of Segments of Gastrointestinal Tract and Partial Pancreatectomy in Male Rats*

	Body weight	Liver		Plasma		Pancreas	Per cent of injected dose in wall and contents			
		Per cent of injected dose in liver	Concentration, as labeled I	Per cent of injected dose in total plasma	Concentration, as labeled I	Concentration, as labeled I	Stomach	Duodenum	Jejunioileum	Colon
	gm.		mg. per 100 gm.		mg. per 100 gm.	mg. per 100 gm.				
Normal controls	298	22.5	1.0	3.1	0.22	0.18	2.0	8.1	39.2	0.7
	269	18.3	1.0	3.4	0.27	0.32	13.2	8.7	20.4	1.0
	352	15.8	0.6	2.7	0.16	0.05	9.1	16.8	34.8	1.3
Average.....	306	18.8	0.9	3.1	0.22	0.18	8.1	11.2	31.4	1.0
Ligated	308	34.8	1.3	8.0	0.56	0.30	1.1	0.4	2.9	1.8
	256	23.2	1.2	5.4	0.45	0.21	1.3	0.2	2.8	0.4
	291	33.9	1.4	7.4	0.55	0.25	1.3	0.5	2.9	1.1
	320	30.8	1.1	7.2	0.54	0.20	1.2	0.4	2.5	1.1
Average.....	293	30.7	1.2	7.0	0.52	0.24	1.2	0.4	2.8	1.1

months before the operation, the animals had been partially pancreatectomized (about 75 per cent of the pancreas being removed). Further-

more, immediately before the injection of radiothyroxine, ligatures were placed about the bile duct, pylorus, duodenojejunal junction, and ileocolic junction. The animals were then intravenously injected with 1.4 mg. of labeled thyroxine and sacrificed 2 hours later (Table IV).

### *Results*

The figures obtained for the localization of labeled thyroxine (Table II) showed first that the radioactivity rapidly disappeared from the blood, since the whole plasma contained only about 1 to 2 per cent of the injected dose of thyroxine 2 hours after injection, and 0.1 per cent 24 hours after injection. Furthermore, only a fraction of this radioactivity was present in a butanol-soluble form, being presumably thyroxine (Table III). The amount found in blood cells was very minute.

The liver fixed a considerable portion of the injected dose; namely, from 9 to 24 per cent at 2 hours after injection. By the 24th hour, there was a moderate decrease to 4 to 8 per cent. At both time intervals, the radioactivity was predominantly in the form of thyroxine. The fixation of thyroxine by the liver occurred rapidly, since 21 per cent of the injected dose was once found in the liver of an animal dying at the end of a 10 minute intravenous injection. It may also be emphasized that the concentration of radioactivity in the liver was greater than in any other organ at 24 hours after injection.

The main result of these experiments was the finding of a very high proportion, usually about 50 per cent, of the injected dose in the various parts of the gastrointestinal tract 2 hours after injection, the most notable concentration being found in the jejunoileum. At 24 hours after injection, thyroxine had almost completely vanished from the gastrointestinal tract, except for the colon in one case. In contrast, at that time interval, the feces showed a very high concentration, since they contained from 66 to 95 per cent of the injected dose. In the jejunoileal and colon-cecal contents as well as in the feces, more than half of the radioactivity was present as thyroxine. On the other hand, almost all of the radioactivity in the stomach was in the form of iodide precipitable by acid silver nitrate.

The kidney contained a moderate concentration of radioactivity as thyroxine, which decreased relatively slowly over a 24 hour period. However, the appreciable amount of radioactivity present in the urine was mostly in the form of iodide. It may be emphasized that the concentration of radioactivity found in secretory glands such as pancreas, submaxillary, etc., at 2 hours after injection had decreased considerably by the 24th hour. This was also true of skin, muscles, and uterus. On the other hand, the concentration of radiothyroxine decreased less rapidly between the 2nd and 24th hours after injection in the lymphatic organs: spleen, thymus, and lymph nodes.

The adrenals, and to a smaller extent the ovaries, also maintained their concentrations of radioactivity until the 24th hour after injection. With the exception of one animal in a recent series (not reported in Table II), no detectable amount of radioactivity could be found in the pituitary gland. In this one animal sacrificed 2 hours after the injection of 3 mg. of radiothyroxine, 0.001 per cent of the injected dose was found in the hypophysis, the concentration ratio being 0.21. The behavior of the thyroid gland was variable. If the animals that received 0.8 mg. of radiothyroxine were compared, 0.09 and 0.06 per cent of the dose was found 2 hours after injection and the values went up to 0.25 per cent at the later time interval.

Counts obtained on the hypothalamic region in the four animals receiving 0.8 mg. were at the limit of statistical significance. In nervous tissue, both isolated gray and white matter showed very low amounts of radioactivity. Miscellaneous organs such as lacrimal glands, lens, chorioid, bladder, vagina, aorta, trachea, testis, bone, etc., showed low counts which in most cases were at the limit of statistical significance.

The second series of experiments was carried out in order to decide in which part of the digestive system thyroxine was excreted during the first 2 hours after injection (Table IV). After separation from the other sections of the intestine by ligation, the jejunioileum contained only from 2 to 3 per cent of the injected dose of radioactivity, instead of 20 to 40 per cent found in the intact control animals. Separations carried out on this organ in the ligated animals showed that most of the radioactivity was present as thyroxine. Similarly low values were observed in the stomach but the radioactivity was mostly present as iodide. The amount and chemical form of the radioactivity present at 2 hours after injection in the large intestine were not affected by the experiment.

Finally, the possibility that an inactive form of thyroxine was being excreted into the gastrointestinal tract was examined by testing the biological activity of butanol extracts of feces from thyroxine-treated animals. Rats were given 5 mg. of thyroxine prepared from diiodothyronine in the usual manner. The feces were collected and extracted with butanol. The butanol was evaporated and the residue assayed on thyroidectomized male rats. The animals treated with this material (Table I) showed a significant increase in oxygen consumption, this increase being somewhat less than that obtained with an amount of synthetic thyroxine of equal iodine content.

#### DISCUSSION

From the outset it must be realized that the doses used in this investigation resulted in concentrations in the organs much above the physiological range. However, current work with physiological amounts of thyroxine (1  $\gamma$  and less) indicated a similar trend of distribution.

The rapid disappearance of radiothyroxine from the blood stream confirmed results previously obtained with thyroid preparations (10-13) and thyroxine (14-16). However, when thyroxine was incubated with blood *in vitro*, none of its activity was lost (17). Therefore, the disappearance of thyroxine from the plasma was not due to its destruction there, but to its withdrawal from the blood by organs and tissues.

The large amounts of radioactivity found in liver and the gastrointestinal tract demonstrated that these organs played a predominant rôle in the disposal of the blood thyroxine. The ligation experiment (Table IV) indicated that the stomach, jejunum, and colon-rectum were able to excrete thyroxine themselves, but only in small amounts. The large amounts found in normal animals must, therefore, be supplied by the accessory digestive glands, liver and pancreas. The rôle of the pancreas is not important, because despite the presence of about 25 per cent of the gland in the partially depancreatized animals, very little thyroxine was found in the duodenum. The liver must, therefore, be the source of the bulk of the intestinal radioactivity. This was also shown by the increased amount of radioactivity which accumulated in the liver when bile excretion was prevented (Table IV). This conclusion was in keeping with the general finding of large amounts of thyroid hormone in the liver after injection of thyroid extract (10, 11, 13, 18), but especially after administration of thyroxine (19) (see also Tables II and IV), as well as with the elimination of thyroxine through the bile reported by other authors (14, 19-23). Thyroxine detoxification in the liver has been shown by the fact that partial hepatectomy enhanced the activity of thyroxine (24). The action of the liver on thyroxine was apparently to excrete some of this substance unchanged through the bile, and possibly to break down some of it, with the liberation of iodide (25).

The excretion of very large amounts of radioactivity in the feces (up to 90 per cent of the injected dose within 24 hours) must be emphasized, since this fact, although mentioned in the literature (19, 21), has not received much attention and the magnitude of the phenomenon had not been realized. The present results demonstrate that thyroxine itself is excreted in the feces, since half of the fecal radioactivity was soluble in butanol and this butanol extract had a definite biological activity (Table I). However, the biological action of a butanol extract containing the same amount of iodine as 50  $\gamma$  of thyroxine was significantly less than that of this amount of thyroxine either prepared by us or obtained from Hoffmann-La Roche. This difference may be due to some inactive iodine compound being extracted together with the thyroxine, or the fecal extract might have a toxic effect causing a diminished metabolic response to thyroxine.

At any rate, the comparison of the 2 hour and 24 hour results (Table II)

indicated that the thyroxine excreted at first into the lumen of the intestine found its way into the feces. This interpretation was complicated by the fact that the gastrointestinal system reabsorbs thyroxine from its lumen, as is shown by the well known efficacy of oral treatment with thyroxine and by experiments demonstrating the resorption within 12 hours of 90 per cent of the thyroxine injected into isolated intestinal loops (21). Consequently, there must be a constant transport of thyroxine into and out of the intestine. That the excretion process predominates is shown by the high thyroxine content of intestinal lumen and feces.

In contrast to fecal excretion, which deals to a large extent with thyroxine itself (Table III), urinary excretion dealt with degradation products of thyroxine, since almost all of the radioactivity in the urine was extracted by butyl alcohol and could be identified as iodide. Reports in the literature state that, after injection of thyroxine into rats, no iodine is found in their urine (19), while in dogs, there was some iodine, but it was not biologically active (14). It may be concluded that there was little or no excretion of thyroxine in the urine, but an appreciable elimination of iodide originating from thyroxine degradation. In contrast, a fair amount of butanol-soluble radioactivity was present in the kidney. The presence of thyroxine in this organ may be best explained by the tubular resorption of thyroxine from the glomerular filtrate.

The organs that fix the radioactivity may be divided into two groups, (a) those showing an initial high concentration which fell rapidly with time, and (b) those in which the concentration decreased relatively slowly with time. In the first group were found the gastrointestinal tract and its associated glands (pancreas, salivary glands, etc.). In the second group were found the liver, lungs, adrenals, ovaries, kidneys, and lymphatic tissue. The organs which are known to be influenced by thyroxine may belong to either category. Thus the first group included the pancreas, which was markedly increased in weight and showed a stimulation of secretion under thyroxine (26), and the gastrointestinal tract and salivary glands, which were apparently unaffected by thyroidectomy or by thyroxine treatment (27). The second group included liver, adrenals, ovaries, and kidneys, which were modified by thyroidectomy or thyroxine treatment, as well as lungs and lymphatic tissue which were not directly affected (27).

In the case of the pituitary gland, a significant amount of radioactivity was detectable in only one animal, the concentration being rather low. This was in accord with recent results in which no iodine could be detected in the human hypophysis and only extremely minute amounts in the beef gland (28). These results indicated that with these doses of thyroxine there was no elective fixation of radioactive thyroxine in the pituitary of the rat, contrary to the results found in the hypophysis of the rabbit (3-5).

In the four animals given 0.8 mg. of radiothyroxine no significant fixation in the hypothalamic region could be detected either.

It is of interest to mention that on adding the amounts of thyroxine recovered by butyl extraction from all organs and excreta, one could calculate that about 65 per cent of the injected dose was still present as thyroxine at 2 hours after injection and 46 per cent at 24 hours. It must be remembered that the figures obtained with the butanol extraction of thyroxine are approximately 15 per cent too low. Therefore, these results roughly indicated that about one-fourth of the thyroxine was destroyed within 2 hours after injection and about one-half within 24 hours after injection. The degradation of thyroxine yielded iodide, as is shown by the presence of large amounts of iodide in urine and gastric juice, in which injected iodide is known to be excreted (29).

The figures obtained for the radioactivity concentration in the thyroid suggested that the concentration increased with time, at least in the animals given 0.8 mg. of thyroxine. It is likely that the iodide gradually released from the breakdown of thyroxine was taken up by the gland. However, the results did not eliminate the less likely possibility that some of the thyroxine entered the gland as such.

#### SUMMARY

1. DL-Thyroxine containing 2 atoms of radioactive iodine in the 3' and 5' positions has been prepared. This material has been shown to be chemically and biologically indistinguishable from commercial DL-thyroxine.

2. This radioactive thyroxine has been administered in large amounts to young female albino rats and its distribution at 2 and 24 hours examined in over forty organs and tissues. In many of these the injected thyroxine has been recovered by *n*-butanol extraction.

3. As early as 2 hours after the administration of labeled thyroxine, not more than 2 per cent of the radioactivity is detectable in the entire volume of circulating plasma, while more than 50 per cent of it can be detected in the gastrointestinal tract, liver, and pancreas, mainly in the form of thyroxine, except in the stomach, where it may be demonstrated as iodide. Other organs showing significant concentrations are the kidneys, lungs, adrenals, ovaries, and lymphatic tissue.

4. At 24 hours after administration of labeled thyroxine, an average of 80 per cent of the injected material may be found in the feces, to a great extent biologically unchanged, while an average of 11 per cent of the dose can be demonstrated as inorganic iodine in the urine. The liver, kidneys, adrenals, ovaries, and lymphatic organs tend to maintain their concentration of radioactivity at this time interval.

5. The route of entry of thyroxine into the gastrointestinal tract was



shown to be mostly via the liver and bile, as ligation of the bile duct and segments of the gastrointestinal tract resulted in an increase in the radioactivity of the liver and the blood, with a corresponding marked decrease in the radioactivity found in the intestine.

6. Approximately 25 per cent of a dose of thyroxine of 0.8 mg. or more is destroyed in 2 hours and 50 per cent in 24 hours. In addition, by 24 hours 80 to 95 per cent of the injected dose has been eliminated from the body.

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# ASPERGILLIC ACID: AN ANTIBIOTIC SUBSTANCE PRODUCED BY *ASPERGILLUS FLAVUS*

## I. GENERAL PROPERTIES; FORMATION OF DESOXYASPERGILLIC ACID; STRUCTURAL CONCLUSIONS

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The discovery by White (1) in 1940 that a strain of *Aspergillus flavus*, growing in surface culture on a tryptone-salt medium, produced a highly bactericidal filtrate from which White and Hill in 1943 (2) were able to isolate the active material in crystalline form added another member to the increasing group of chemically and biologically interesting antibiotic compounds. Because the mode of isolation indicated that it was an acidic compound, it was named *aspergillic acid*.<sup>1</sup> White and Hill reported studies of its antibiotic properties and its animal toxicity and listed a few of its physical and chemical properties.

Using a selected variant of White's strain of *Aspergillus flavus*, Jones, Rake, and Hamre (8) were able to obtain increased production of aspergillic acid by the mold, and a modification of the isolation method of White and Hill was devised by Menzel, Wintersteiner, and Rake (9). A preliminary report summarizing the results of the investigation of the structure of aspergillic acid was made in 1944 by Dutcher and Wintersteiner (10). Other reports have appeared concerning the production of antibacterial substances by *Aspergillus flavus* in surface culture, but it is probable that in most cases the substance is aspergillic acid or a mixture of aspergillic acid and the higher melting hydroxyaspergillic acid discovered by Menzel, Wintersteiner, and Rake (9) in culture filtrates of *Aspergillus flavus* grown on a medium containing brown sugar. Glister (11) isolated an active substance

<sup>1</sup> There is some confusion in the literature concerning the naming of this substance. White, the discoverer, refrained from calling it "aspergillin" in the manner after penicillin because of the preempting of this name by the pigment of *Aspergillus niger*. Later workers have been less fastidious, however, and have applied the term "aspergillin" to active materials from *Aspergillus flavus* and other *Aspergilli*; Bush and Goth (3) initially called their substance aspergillin but later recognized it as aspergillic acid; Stanley (4) designated the active substance produced by an unidentified *Aspergillus* strain as "aspergillin" but has subsequently reported (5) that this compound is identical with gliotoxin; still another crude product, obtained from *Aspergillus fumigatus* by Soltys (6), has been called "aspergillin" but it appears likely from the latest report (7) that this product is helvolic acid (fumigacin). ■

from the culture filtrate of an unidentified mold. The identity of this substance with aspergillic acid was shown by comparison of the physical properties and biological activity (9). Bush and Goth (3) and Bush, Dickison, Ward, and Avery (12) have examined the material produced by a strain of *Aspergillus flavus* and report that in the main the active substance is aspergillic acid. A recent note by Salvin (13) reports a crystalline antifungal and antibacterial substance produced by a strain of *Aspergillus flavus* which appears to be similar to but not identical with aspergillic acid.

The production and isolation of aspergillic acid has been carried out in various ways, which are summarized in the experimental section. The present paper describes in greater detail the physical properties of pure aspergillic acid and presents some of its unusual chemical reactions and the conclusions concerning its structure which have been made on the basis of these findings.

Pure aspergillic acid crystallizes as clusters of pale yellow, elongated rods, possessing a characteristic odor suggestive of black walnuts; the melting point determined in a capillary tube is 93°; the specific rotation in ethanol is  $[\alpha]_D^{24} = +12^\circ (\pm 3^\circ)$ . Aspergillic acid is soluble in most organic solvents, but has very low solubility in water. Analysis and molecular weight determination established the formula of aspergillic acid as  $C_{12}H_{20}O_2N_2$ . Its acidic nature was demonstrated by its solubility in dilute sodium hydroxide or sodium carbonate solution, and it could be titrated in alcoholic solution with phenolphthalein as indicator. Electrometric titration showed the  $pK'_a$  to be 5.3. The silver salt was obtained as colorless plates from alcohol and the copper salt crystallized as grass-green rectangular leaflets. The substance also has weakly basic properties, as is demonstrated by the formation of a crystalline hydrochloride in anhydrous medium and the formation of a crystalline salt with 3,5-dinitrobenzoic acid.

The ultraviolet absorption spectrum<sup>2</sup> of aspergillic acid in various solvents is shown in Fig. 1.

The examination of this antibiotic substance revealed the following properties: (1) It contained no methoxyl or methyl imide groups but yielded 2 moles of acetic acid in the Kuhn-Roth determination of C-methyl groups. (2) It was indifferent to carbonyl reagents, except that it formed a crystalline salt with phenylhydrazine in alcoholic solution. (3) It could not be acylated by any of the usual procedures. (4) It reacted in ethereal solution with diazomethane with the formation of a neutral non-crystalline product. (5) It gave an intense red color upon treatment of a methanolic solution

<sup>2</sup> All of the ultraviolet absorption spectra given in this paper were determined by Dr. Nettie Coy of the Squibb Biological Laboratories, for whose cooperation and interest in interpreting the ultraviolet absorption data obtained during the investigation of this compound the author expresses sincerest gratitude.

with ferric chloride. (6) It did not react with bromine in carbon tetrachloride or glacial acetic acid solution, but reacted readily with bromine water. (7) It did not reduce Fehling's or neutral permanganate solution. (8) It was indifferent towards phenyl isocyanate. (9) It was recovered unchanged after long periods of refluxing with strong alkali or acid. (10) It did not react with nitrous acid.

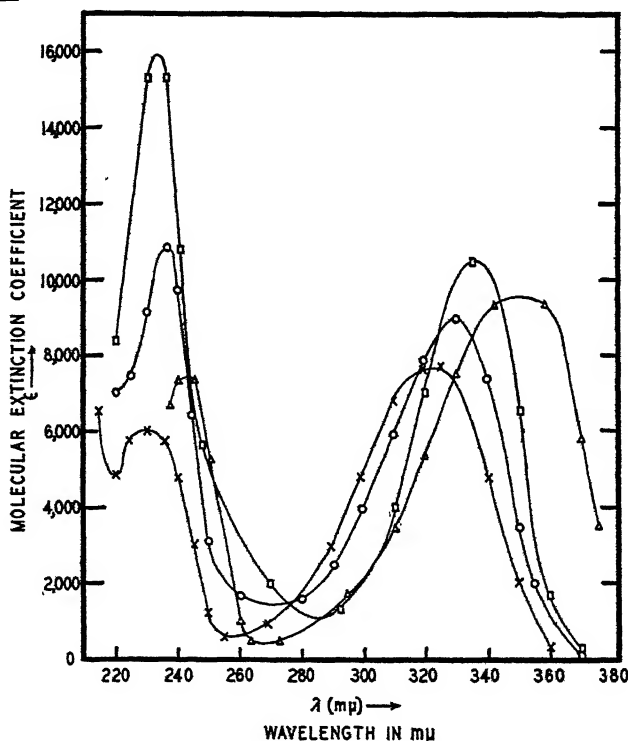


FIG. 1. Ultraviolet absorption spectrum of aspergillic acid in ethanol (O), pH 7.0 buffer (□), hexane (X), 0.1 N HCl (Δ).

It was at first felt that the 2 oxygen atoms of aspergillic acid were present as a carboxyl group, but the attempt to decarboxylate it by means of dry distillation in the presence of copper chromite catalyst (14) led unexpectedly to the formation of a neutral, optically active product with the same number of carbon and hydrogen atoms but with 1 less oxygen atom,  $C_{12}H_{20}N_2O$ . This product possessed essentially the same ultraviolet absorption spectrum as aspergillic acid, as is shown in Fig. 2. Such a reaction could not be explained by the existence of a carboxyl group in aspergillic acid and the acidity must therefore be due to some other grouping. Two of the reac-

tions previously mentioned, the deep red coloration with ferric chloride and the formation of a green cupric salt, suggested that aspergillic acid might possess a hydroxamic acid type of grouping as is shown in Formula I.

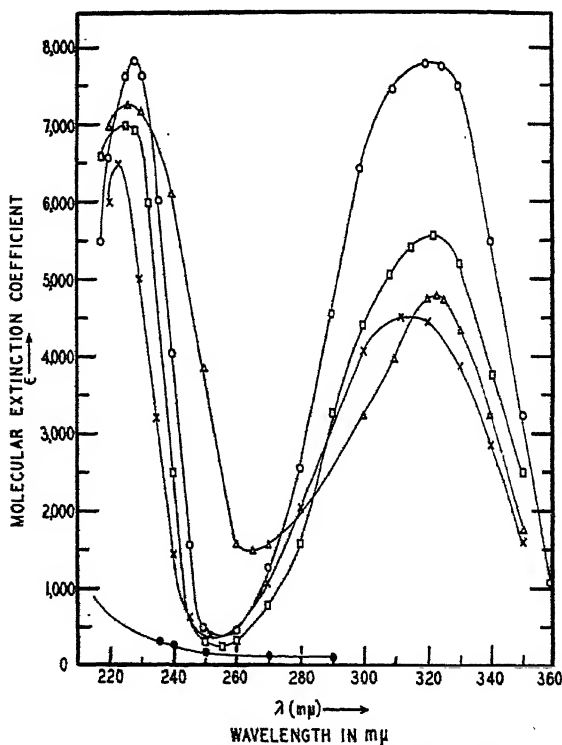
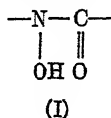


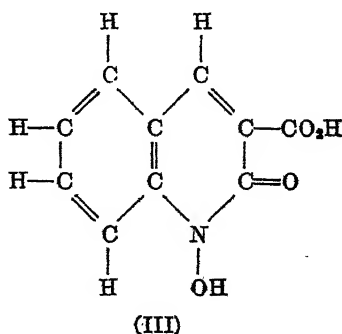
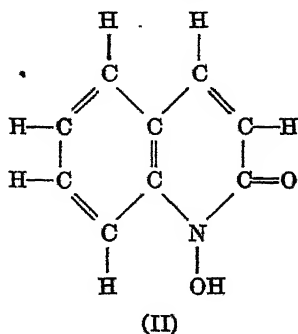
Fig. 2. Ultraviolet absorption spectrum of desoxyaspergillic acid (O), tetrahydrodesoxyaspergillic acid (●), 2-aminopyrazine (Δ), 2-hydroxypyrazine (X), 1-methyl-2-hydroxypyrazine (□); all determined in ethanol.

To confirm this postulation, and in the hope of obtaining better yields, other methods of preparation of the neutral product,  $C_{12}H_{20}N_2O$ , which was called *desoxyaspergillic acid*, were sought. It is well known that hydroxyl groups on nitrogen are easily replaced by hydrogen by a variety of reducing agents, and, hence, several reductions of aspergillic acid were tried. No reduction was effected by catalytic hydrogenation in either alcohol or acetic acid, nor was there any reduction by stannous chloride in concentrated hydrochloric

acid solution. However, when aspergillic acid was treated with zinc in acetic acid solution, reduction did occur, as was shown by the loss of the yellow color and the disappearance of the ferric chloride color reaction. The product isolated from this reduction was not desoxyaspergillic acid, however, but a low melting, weakly basic substance which formed a crystalline hydrochloride. The analysis of this compound agreed with the formula  $C_{12}H_{24}N_2O \cdot HCl$ . It was therefore the hydrochloride of *tetrahydrodesoxyaspergillic acid*, the zinc and acid conceivably having reduced a pair of double bonds in aspergillic acid in addition to removing the hydroxyl group from the nitrogen atom. This reduction of the unsaturated linkages of aspergillic acid was further attested by the absence of any specific absorption in the ultraviolet by tetrahydrodesoxyaspergillic acid (Fig. 2). In contradistinction to aspergillic acid, desoxyaspergillic acid could be reduced catalytically with platinum and hydrogen to yield, by the absorption of 2 moles of hydrogen, tetrahydrodesoxyaspergillic acid, identical with the product obtained by zinc and acetic acid reduction of aspergillic acid.

Two methods were found, however, which reduced aspergillic acid only as far as desoxyaspergillic acid; the first was reduction with hydriodic acid in acetic acid solution and the second was the treatment with hydrazine at elevated temperatures. Both of these reactions also were found capable in model experiments of effecting the reduction of other hydroxamic acids to the corresponding amides.

The stability of both aspergillic acid and desoxyaspergillic acid to acidic and alkaline hydrolysis indicated that the hydroxamic acid grouping could not be of simple aliphatic nature, since such groupings are rather readily hydrolyzed or undergo rearrangement. It was necessary therefore to conceive of this grouping in aspergillic acid as part of a heterocyclic system. Only two other compounds of this type have been reported in the chemical literature; these are the oxycarbostyryl of Friedländer and Ostermaier (15), Formula II, and the carboxyoxycarbostyryl of Heller and Wunderlich (16), Formula III. The marked similarity of the properties of aspergillic acid

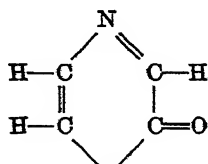


to those described for the above compounds offers convincing confirmatory evidence for the presence of such a grouping in the molecule in question.

The nature of the carbon and nitrogen atoms other than those of the hydroxamic acid group of aspergillic acid remained to be demonstrated. The function of the 2nd nitrogen atom could not be that of a primary amine in view of the weak basicity and failure to react with nitrous acid, nor could it be substituted by lower alkyl groups in view of the absence of any volatile halides in the methyl imide determination. Thus it appeared likely that this nitrogen too was present in a heterocyclic system. Furthermore, it followed from a consideration of facts already mentioned (indifference to potassium permanganate and bromine, resistance to hydrolysis, formation of a tetrahydro derivative) that the two double bonds demonstrable by reduction must be present in a six-membered aromatic system, *i.e.* in a diazine nucleus.

The ultraviolet absorption maximum at 325  $m\mu$  which both aspergillic and desoxyaspergillic acid exhibited was not characteristic of any of the monooxypyrimidines, none of which had maxima beyond 290  $m\mu$ . As no ultraviolet data for oxypyridazines or oxypyrazines could be found in the literature, the synthesis of model compounds was undertaken.

On the assumption that a pyrazine structure, rather than a pyridazine, was more probable for a naturally occurring substance,  $\alpha$ -pyrazone (pyrazinol-2, hydroxypyrazine) was prepared from 2-aminopyrazine, and its ultraviolet absorption spectrum was obtained. As is shown in Fig. 2, this absorption curve resembles closely that of aspergillic acid, and in view of this similarity a tentative structure for aspergillic acid was formulated which incorporated the features of a hydroxamic acid in a pyrazine ring. Such a structure, shown in Formula IV, would account for the 2 nitrogen atoms in aspergillic acid, 1 in the hydroxamic acid group and the other as a weakly basic heterocyclic one. Both oxygen atoms are accounted for by

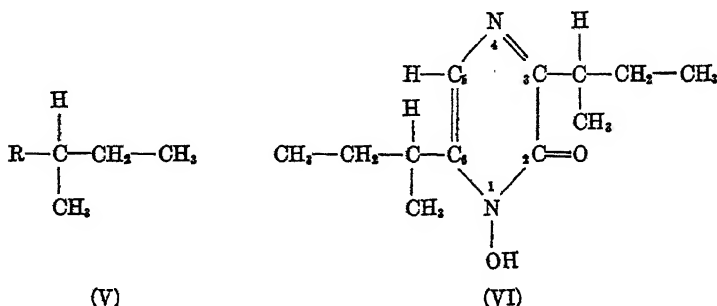


OH  
(IV)

the hydroxamic acid group, and the  $\alpha$ -pyrazone nucleus accounts for the ultraviolet absorption as well as for the two double bonds indicated by the formation of the tetrahydro product. Subtraction of the elements of this

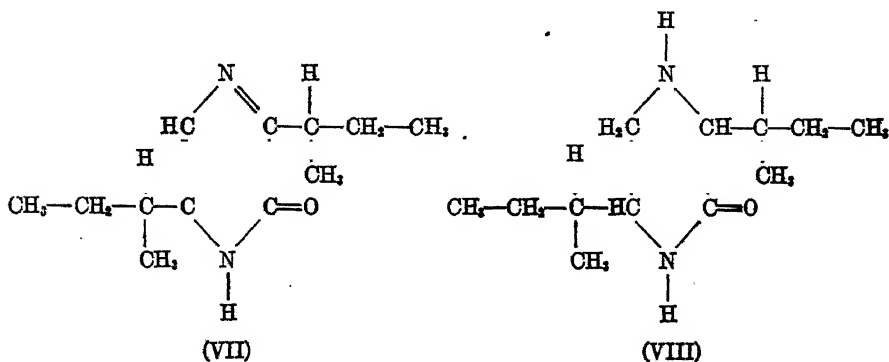
nucleus,  $C_4H_4N_2O_2$ , from those of aspergillic acid,  $C_{12}H_{20}N_2O_2$ , leaves simply an aliphatic residue,  $C_8H_{16}$ .

In the light of reactions which will be described in detail in the following paper, it appeared that there were two alkyl groups attached to the  $\alpha$ -pyrazone nucleus, since only one position was free for substitution. The fact that aspergillic acid is optically active required that at least 1 asymmetric carbon atom be present in the side chains. The simplest optically active aliphatic grouping, the secondary butyl, requires 4 carbon atoms arranged as in Formula V. Considering biogenetic possibilities, the working hy-



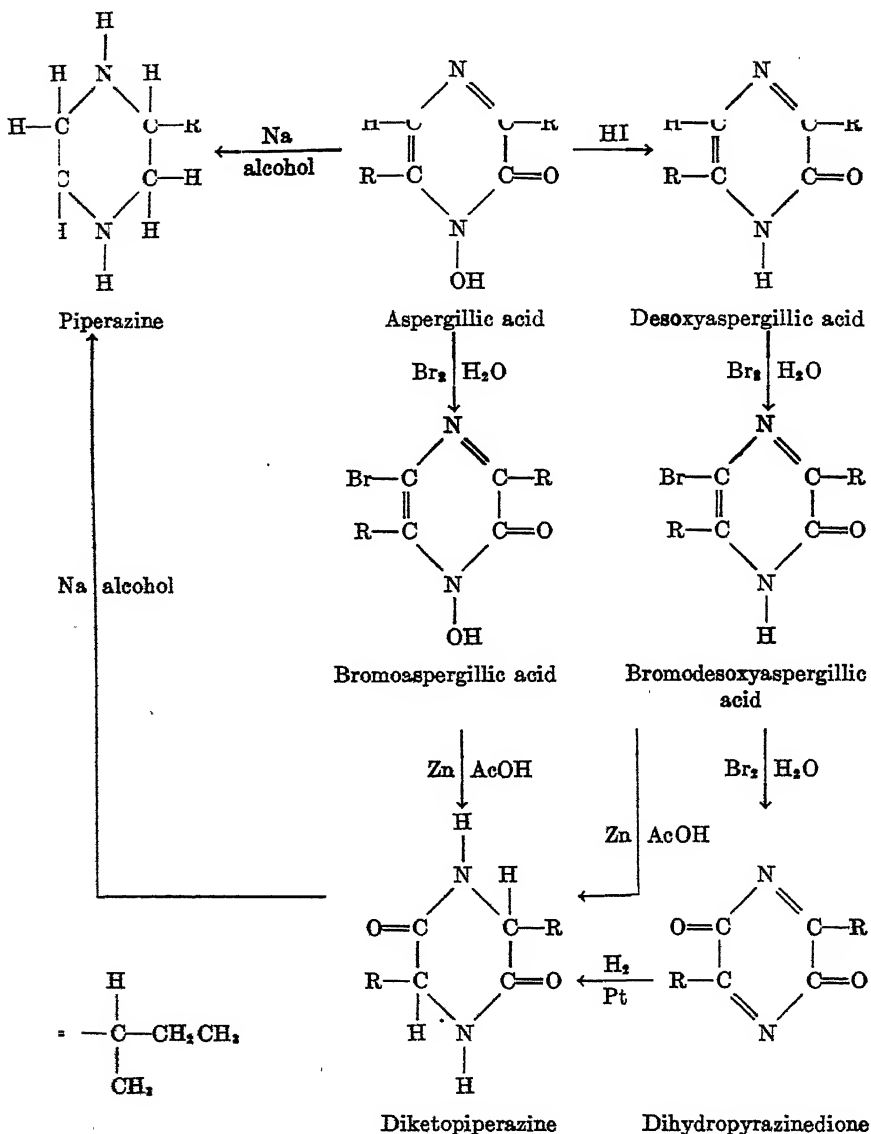
pothesis was adopted that the side chains were both secondary butyl groups (a feature which would satisfy the experimentally determined requirement for two C-methyl groups) and were symmetrically placed at positions 3 and 6, as shown in Formula VI.

Evidence verifying this formula as representing the structure of aspergillic acid was obtained by two routes: first through substitution of the hydrogen atom at position 5 by bromine and conversion of the bromoaspergillic acid to a 2,5-diketopiperazine, as shown in the accompanying scheme, and second by total reduction of aspergillic acid with sodium and alcohol to a piperazine derivative, also shown in the scheme. The structures of





these two degradation products it was found possible to confirm by comparison with the corresponding synthetic products.



On the basis of Formula VI for aspergillilic acid, desoxyaspergillilic acid would be represented by Formula VII and tetrahydrodesoxyaspergillilic acid by Formula VIII. Subsequent to the announcement of the structure

of aspergillic acid and desoxyaspergillic acid (10), Newbold and Spring (17), in the course of the synthesis of various oxypyrazines, prepared 2-amino-3,6-di-*sec*-butylpyrazine and from it, 2-hydroxy-3,6-di-*sec*-butylpyrazine, Formula VII. Because of their inability to resolve the mixture of stereoisomers, direct comparison with a sample of the optically active desoxyaspergillic acid from the natural product was not possible, but they concluded that the compounds possessed the same structure in view of the close similarity of properties such as solubility and ultraviolet absorption spectrum.

#### DISCUSSION

The occurrence of a hydroxamic grouping in a natural product has not been previously reported. The hypothesis has been advanced (18, 19) that formhydroxamic acid, the product of activated formaldehyde and inorganic nitrite, is an intermediate in the phytosynthesis of  $\alpha$ -amino acids and alkaloids, but no experimental evidence indicating that such is the case has been forthcoming. A hydroxylamino derivative, canavanine, has been isolated from the jack bean (20) and amine oxides are known to occur in various fish muscle extracts, but that is about the extent of such compounds so far discovered.

The fact that a hydroxamic acid derivative such as aspergillic acid should possess considerable toxicity both for microorganisms and higher animals is not unexpected, since it has long been known and frequently observed that hydroxylamine and its derivatives are highly potent cell poisons. That the antibacterial activity of aspergillic acid is due to the hydroxylated nitrogen atom is shown by the lack of antibiotic activity of desoxyaspergillic acid. Several synthetic hydroxamic acids have been tested and found to possess considerable antibacterial activity. Details of this work will be published in a subsequent paper.

#### EXPERIMENTAL

*Production of Aspergillic Acid*—The methods described in the literature for the production of aspergillic acid are essentially variants of the original procedures used by White and Hill (2). These authors reported that, while their strain of *Aspergillus flavus* would grow on a wide variety of media, it only produced significant amounts of aspergillic acid on Difco tryptone, peptone, or corn steep liquor media. Since 2 per cent Difco tryptone with 0.5 per cent sodium chloride gave the highest titers, this was adopted as their standard medium. The yields reported by White and Hill varied from 5 to 70 mg. of crude crystalline material per liter of culture filtrate. Jones, Rake, and Hamre (8) employed the same medium for the production of aspergillic acid but found that higher titers could be obtained by adding

2 to 4 per cent of brown sugar. With their strain of *Aspergillus flavus* the yields of crystalline aspergillic acid from the tryptone and salt medium varied between 120 and 253 mg. per liter; from the brown sugar-containing medium the yield of crystalline material reached as high as 400 mg. per liter. However, this latter material was shown by Menzel, Wintersteiner, and Rake (9) to consist chiefly of a product which melted at 145–146° as compared with 93° for the aspergillic acid obtained by White and Hill. Furthermore, while the formula for the aspergillic acid melting at 93° was  $C_{12}H_{20}N_2O_2$ , analysis of the high melting compound showed that it possessed the formula  $C_{12}H_{20}N_2O_3$ . The antibacterial activity of this high melting compound, called hydroxyaspergillic acid, was only one-tenth to one-third that of aspergillic acid. Bush, Dickson, Ward, and Avery (12), in investigating the antibiotic substances produced by a strain of *Aspergillus flavus* Link, found that they obtained the best titers on 2 per cent Difco peptone-2 per cent lactose medium. They isolated, by an extraction procedure, material which also appeared to be a mixture of aspergillic acid and hydroxyaspergillic acid. Their typical culture filtrate was stated to possess one-half the antibacterial potency of a standard solution containing 1 mg. of purified aspergillic acid per ml. and hence a concentration in the filtrate of approximately 500 mg. per liter. The recovery in the form of crude acid was about 55 per cent.

Since the separation of aspergillic acid and hydroxyaspergillic acid, to be described in a later paper, is quite difficult and laborious, the production of material for structural investigation was confined to those media which contain no sugar and on which the mold produces only aspergillic acid of melting point 93°. It has been found that in addition to the Difco tryptone-sodium chloride medium a yeast extract-glycerol medium will produce aspergillic acid of melting point 93° in consistently good yields of from 800 to 1000 mg. per liter (21).

During the course of the investigation of the production of aspergillic acid on different media by Mr. Carl Woodward, Jr., of the Bacteriological Production Laboratories, E. R. Squibb and Sons (21), a spectrophotometric method for quantitative assay, with use of the Beckman ultraviolet spectrophotometer, was worked out in collaboration with Dr. Nettie Coy of the Squibb Biological Laboratories. It was found that with the various media tested no substances other than aspergillic acid were produced which absorbed in the ultraviolet region 300 to 350  $m\mu$ . The position and intensity of the aspergillic acid band was determined at pH 8, which is approximately that of the medium at maximum concentration. The maximum at this pH is at 336  $m\mu$  with  $\epsilon = 10,500$  as compared with 325  $m\mu$ ,  $\epsilon = 8900$  in ethanol. The density of an unknown solution can therefore be multiplied by the factor 2.13 to give the concentration of aspergillic acid in mg. per ml.

The spectrophotometric values were found to parallel the bioassay values in all cases in which only aspergillic acid of melting point  $93^{\circ}$  was formed. Under other conditions in which the organism produces hydroxyaspergillic acid as well as aspergillic acid (9), the spectrophotometric measurement gives the correct sum of both constituents because the absorption bands of the two products are identical but the bioassay value will be considerably lower, depending upon the proportion of hydroxyaspergillic acid present, since this product has only about one-tenth the antibiotic activity of aspergillic acid.

*Isolation and Purification of Aspergillic Acid*—The most satisfactory procedure for the isolation and purification of aspergillic acid involves extraction of the acidified culture filtrate with chloroform, concentration of the chloroform to a small volume, reextraction from the chloroform solution with sodium carbonate solution, and precipitation from the aqueous solution by acidification. The crude acid thus obtained is best purified by dissolving in boiling hexane and filtering from brown, amorphous, insoluble material. Concentration of the hexane solution leads to the separation of nearly pure aspergillic acid of melting point  $90-93^{\circ}$ . Further crystallizations from acetone or methanol, with charcoal, are carried out if necessary.

The analysis and description of aspergillic acid has been given in previous reports (2, 9, 10) and is given here with further characterization only for the sake of completeness.

As originally described and photographed by White and Hill (2), aspergillic acid crystallizes as clusters of pale yellow, elongated plates or rods, and melts<sup>3</sup> quite sharply at  $93^{\circ}$  when uncontaminated by the high melting hydroxyaspergillic acid. It is readily soluble in most organic solvents but only to the extent of about 1 mg. per ml. in water.

Titration in alcohol with phenolphthalein as indicator shows a neutralization equivalent of 224. When titrated electrometrically, the same equivalent weight is obtained; the  $pK'_a$  is 5.3.  $[\alpha]_D^{24} = +13.4^{\circ}$  in ethanol,  $c = 0.85$ ;  $[\alpha]_D^{24} = +18.5^{\circ}$  in 1 N NaOH,  $c = 1.05$ .

*Analysis*<sup>4</sup>— $C_{12}H_{20}N_2O_2$  (224.3)

Calculated. C 64.25, H 8.98, N 12.49

Found. " 64.42, " 8.96, " 12.73, mol. wt. (Rast) 220  
" 64.10 " 8.80 " 12.50

No methoxyl or methyl imide groups were demonstrable by the methods of Zeisel and Friedrich. Kuhn-Roth C-methyl determination yielded 2.10

<sup>3</sup> All melting points were determined in a capillary tube in an oil bath and are uncorrected.

<sup>4</sup> The analyses reported in this paper, including the Kuhn-Roth C-methyl and Friedrich N-methyl determinations, were carried out by Mr. J. F. Alicino of the Squibb Institute.

moles of acetic acid per mole of aspergillic acid. By the same method isoleucine yielded 1.0 mole of acetic acid.

*Copper Salt*—The copper salt of aspergillic acid is prepared by treating an alcoholic solution with alcoholic cupric acetate. The addition of water to the solution causes the salt to crystallize out as grass-green rectangular plates, m.p. 198–199°.

*Analysis*— $\text{Cu}(\text{C}_{12}\text{H}_{19}\text{N}_2\text{O}_2)_2$

Calculated. C 56.6, H 7.45, N 11.0, Cu 12.50

Found. " 56.7, " 7.49, " 11.0, " 11.60

The addition of copper acetate or copper sulfate solution to the culture filtrate yields an amorphous precipitate of the copper salt of aspergillic acid which can be filtered off, washed, and recrystallized. The copper salt is soluble in ethanol, methanol, acetone, dioxan, and ether but highly insoluble in water or aqueous solvents. It is soluble in 5 N hydrochloric acid but does not precipitate by dilution to 1 N strength. The extraction of this solution with ether yields pure aspergillic acid of correct melting point.

*Silver Salt*—The silver salt of aspergillic acid is prepared by treating an alcoholic solution with alcoholic silver nitrate or with aqueous silver sulfate solution. The salt is obtained as colorless platelets, which darken at 170°, blacken at 186°, and decompose at 190°.

*Analysis*— $\text{C}_{12}\text{H}_{19}\text{N}_2\text{O}_2\text{Ag}$ . Calculated, Ag 32.6; found, Ag 32.7

*Phenylhydrazine Salt*—In a preliminary observation to determine whether aspergillic acid had a carbonyl group, an alcoholic solution was treated with 1 equivalent of phenylhydrazine base and after standing for  $\frac{1}{2}$  hour was treated with water until turbid. Long colorless needles quickly separated out and were recrystallized from dilute alcohol, m.p. 99.5°. Yield, quantitative. The analysis showed this to be the phenylhydrazine salt rather than a phenylhydrazone.

*Analysis*— $\text{C}_{12}\text{H}_{19}\text{N}_2\text{O}_2 \cdot \text{C}_6\text{H}_5\text{N}_2\text{H}_2$

Calculated. C 65.00, H 8.43, N 16.8

Found. " 64.65, " 8.46, " 16.92

*3,5-Dinitrobenzoic Acid Salt*—During efforts to prepare a 3,5-dinitrobenzoate of aspergillic acid by reaction with the acid chloride in pyridine, it was observed that two crystalline products could be isolated. One had a melting point of 166–168° and the other melted at 123° after recrystallization from hot water or dilute ethanol. The solubility of these products in hot water suggested that they were salts, and investigation showed this to be the case. The compound melting at 166–168° could be prepared from equivalent amounts of pyridine and 3,5-dinitrobenzoic acid and that melting at 123° could be obtained by mixing equivalents of aspergillic acid and

3,5-dinitrobenzoic acid in ethanol and precipitating with cold water. The aspergillic acid salt could be titrated in methanolic solution with 0.01 N NaOH solution; 56.9 mg. required 26.0 ml. just to turn phenolphthalein indicator pink. Neutralization equivalent found, 219; calculated for  $C_{12}H_{20}N_2O_2 \cdot C_7H_4N_2O_6$  (mol. wt. 436), neutralization equivalent 218.

*Aspergillic Acid Hydrochloride*—The hydrochloride of aspergillic acid is obtained when an ethereal solution is treated with gaseous hydrogen chloride. The colorless precipitate can be recrystallized from absolute ethanol but hydrolyzes in aqueous solution to yield free aspergillic acid. Aspergillic acid hydrochloride has a melting point of  $182^\circ$  with gradual decomposition over a wide range.

*Analysis*— $C_{12}H_{20}N_2O_2 \cdot HCl$

Calculated. N 10.75, Cl 13.6

Found. " 11.03, " 13.8

*Aspergillic Acid Methyl Ether*—An ethereal solution of aspergillic acid reacts rapidly with diazomethane to yield a neutral oily product that does not crystallize. Distillation under high vacuum yields a colorless syrup which on analysis corresponds with the methyl ether.<sup>5</sup>

*Analysis*— $C_{12}H_{22}N_2O_2$

Calculated. C 65.49, H 9.31, N 11.76,  $CH_3O$  13.02

Found. " 65.34, " 9.21, " 11.70, " 13.01 (Zeisel)

This product is readily soluble in the usual organic solvents and insoluble in water or dilute alkali. The methanolic solution gives no coloration with ferric chloride solution. It possesses insignificant bacteriostatic activity. When an alcoholic solution is slightly acidified and allowed to stand for several hours, the coloration with ferric chloride becomes positive, indicating hydrolysis to aspergillic acid. Because of this ready hydrolysis it was found impossible to prepare a crystalline hydrochloride or hydrobromide of the methyl ether.

The benzyl ether of aspergillic acid was prepared similarly from phenyl-diazomethane but could not be crystallized.

*Formation of Desoxyaspergillic Acid*—This product has been prepared from aspergillic acid in several ways.

1. *Dry Distillation with Copper-Chromite Catalyst*—An intimate mixture of 500 mg. of aspergillic acid and 5 mg. of copper chromite (14) was placed in a distilling flask and heated with a free flame. A stream of nitrogen was passed through the flask during distillation and the escaping gases bubbled through a trap containing barium hydroxide solution. After the mass had fused, water vapors began to condense in the series of collecting bulbs.

<sup>5</sup> This analysis was carried out on material prepared and purified by Dr. Menzel (cf. (9)).

The water was driven from these by gentle flaming. Some precipitate began to form in the baryta trap. A colorless liquid was then distilled over into the bulbs where it crystallized on cooling. Some carbonization occurred in the flask. The amount of barium carbonate which formed weighed only 70 mg. and hence showed that no appreciable decarboxylation had occurred. The solid material in the bulbs was washed out with benzene, which on evaporation yielded a crystalline residue weighing 294 mg. Recrystallization from aqueous ethanol and treatment with charcoal yielded 148 mg. of colorless needles, m.p. 102°. A mixed melting point determination with aspergillic acid showed liquefaction over the range 68–78°.

*Analysis*— $C_{12}H_{20}N_2O$  (208)

Calculated. C 69.20, H 9.62, N 13.45

Found. " 69.34, " 9.63, " 13.56, mol. wt. (Rast) 207

$[\alpha]_D^{24} = +15.3^\circ$  in ethanol,  $c = 0.5$ ;  $[\alpha]_D^{24} = +10.2^\circ$  in ethanol,  $c = 0.74$ ;  $[\alpha]_D^{24} = +18.8^\circ$  in methanol,  $c = 2.17$ .

A similar formation of desoxyaspergillic acid was found to occur when inadvertently a crude batch of precipitated aspergillic acid, probably not washed free of sulfuric acid, was heated for several hours above its melting point. From the resulting tarry mass the only recoverable product was desoxyaspergillic acid. Purified aspergillic acid, however, may be sublimed unchanged in high vacuum. Desoxyaspergillic acid distills at 305–310° at 760 mm. and at 197–199° at 10 mm. pressure.

*2. Reduction of Aspergillic Acid with Hydriodic Acid and Red Phosphorus*—500 mg. of aspergillic acid were dissolved in 15 ml. of glacial acetic acid and added to a mixture of 150 mg. of red phosphorus, 50 mg. of iodine, and 5 ml. of glacial acetic acid. The solution was refluxed for 3 hours and then filtered hot through an asbestos pad into 80 ml. of cold water containing 1.0 gm. of sodium bisulfite. The solid which precipitated was filtered off, washed with cold water, and dried in a vacuum desiccator. The yield was 330 mg., representing 71 per cent of the theoretical. A sample recrystallized from aqueous ethanol showed a melting point of 102° and gave no depression in a mixed melting point determination with the material obtained by dry distillation.

*3. Reduction of Aspergillic Acid by Hydrazine*—An alcoholic solution containing 2.0 gm. in 10 ml., to which were added 5.0 ml. of 85 per cent hydrazine hydrate, was sealed in a bomb tube and heated at 100° for 12 hours. The solution was concentrated in a vacuum and the residue taken up in dilute ethanol and acidified with hydrochloric acid. From the solution there slowly deposited 952 mg. of nearly pure desoxyaspergillic acid, m.p. 101°. A further crop of 400 mg. was obtained on concentration of the solution. A third crop of 150 mg. raised the yield to 81 per cent of the theoretical.

*Properties of Desoxyaspergillic Acid*—Desoxyaspergillic acid is only weakly acidic; it dissolves in 10 per cent sodium hydroxide solution but cannot be titrated. It does not give a copper salt or any trace of coloration with ferric chloride solution. It is more soluble in hot water than is aspergillic acid and may be recrystallized from this solvent. It is correspondingly more basic than aspergillic acid; it is readily soluble in 1.0 N hydrochloric acid and the hydrochloride may be obtained on concentration of the solution. Recrystallized from ethanol, the hydrochloride of desoxyaspergillic acid melts at 207°. The crystalline hydrobromide melts at 250–252° with decomposition.

*Analysis*— $C_{12}H_{20}N_2O \cdot HBr$ . Calculated, N 9.68; found, N 9.58

The nicely crystalline salt prepared from desoxyaspergillic acid and 3,5-dinitrobenzoic acid has a melting point 137–138° after softening at 135°.

Like aspergillic acid, desoxyaspergillic acid is remarkably stable towards numerous reagents. Vigorous treatment with alkaline reagents, such as  $Ba(OH)_2$  and KOH, or acidic reagents, including strong HCl,  $HNO_3$ , and  $H_2SO_4$ , fails to hydrolyze either of these compounds.

Desoxyaspergillic acid was found to be without antibiotic activity in the usual tests, but when a concentration of 3 mg. per ml. was prepared in the medium routinely used for the growth of *Aspergillus flavus*, the germination of the spore inoculum was completely inhibited. This experiment was tried to determine whether the mold would convert desoxyaspergillic acid to aspergillic acid during growth. In view of the observed inhibitory activity on the spores it is planned to test this reconversion capacity of the mold by reflooding a mature mycelial mat with media containing desoxyaspergillic acid. Reflooding experiments with media containing isoleucine indicated no direct utilization of this amino acid for the formation of aspergillic acid.<sup>6</sup>

*Desoxyaspergillic Acid Methiodide*—Whereas aspergillic acid reacted very sluggishly with methyl iodide, desoxyaspergillic acid combined with 1 mole to yield a nicely crystalline methiodide. Hofmann and Emde degradations of this product could not be carried to completion. 160 mg. of desoxyaspergillic acid readily dissolved in 0.5 ml. of methyl iodide. The

<sup>6</sup> Speculation as to the probable biochemical precursor of aspergillic acid readily turned upon isoleucine, since 2 such amino acid residues would yield the necessary carbon and nitrogen skeleton and, as will be described in a subsequent paper, an isoleucine anhydride (3,6-di-sec-butyldiketopiperazine) was obtained in the degradation of aspergillic acid. Furthermore, the presence of free isoleucine in an *Aspergillus* had been reported by Woolley and Peterson (22). Jones and Rake (unpublished work) tested isoleucine in different media and in varying concentrations but obtained no results indicating the direct utilization of this amino acid for the formation of aspergillic acid.



solution became deep red at 50° at which it was held in a sealed tube for 1 hour. On cooling, crystals separated out. The excess methyl iodide was evaporated off and the residue recrystallized from ethanol by the addition of ether. The clusters of orange-yellow plates so obtained weighed 100 mg. and melted to a red liquid at 169°. The mother liquor yielded a nearly equal amount of unchanged desoxyaspergillic acid. Recrystallization of the orange-yellow plates from methanol-ether mixture gave very pale yellow, silky platelets of the same melting point.

*Analysis*— $C_{12}H_{22}N_2OI$  (350)

Calculated. C 44.55, H 6.57, N 8.00

Found. " 44.54, " 6.41, " 7.99

This methiodide is very soluble in methanol, ethanol, and acetone and readily soluble in water. In the presence of sodium acetate and reduced platinum oxide it took up 2 moles of hydrogen very readily to yield a tertiary base but the latter could not be induced to crystallize.

*Tetrahydrodesoxyaspergillic Acid. Zinc and Acetic Acid Reduction of Aspergillic Acid*—2 gm. of aspergillic acid were dissolved in 25 ml. of glacial acetic acid and 25 ml. of warm water added; zinc dust was added in three 1 gm. portions during 5 hours of refluxing. The solution was cooled, diluted with 150 ml. of water, and treated with hydrogen sulfide until no more zinc sulfide precipitated. After filtration of the zinc sulfide the solution was neutralized with sodium hydroxide and extracted with three 100 ml. portions of ether. The ether was washed with water, dried over anhydrous sodium sulfate, and evaporated *in vacuo*, yielding a syrupy residue which weighed 0.944 gm. This residue partially crystallized on standing and the solid was separated with cold acetone. The colorless material was extremely soluble in most organic solvents and in water but could be recrystallized, although with considerable loss, from a concentrated acetone solution. The needles melted at 87–89° after softening at 77° and forming a gel at 84°. When the ethereal solution was treated with dry HCl, a gelatinous hydrochloride separated out which, after drying, was crystallized from ethanol by the addition of ether. Colorless prisms were obtained which melted at 260° after darkening at 235°.

*Analysis*— $C_{12}H_{24}N_2O \cdot HCl$

Calculated. C 58.00, H 10.07, N 11.28, Cl 14.28

Found. " 58.06, " 10.25, " 11.26, " 14.24

$[\alpha]_D^{24} = +4.23^\circ$ ,  $c = 1.18$  in water.

*Catalytic Hydrogenation of Desoxyaspergillic Acid*—Since the composition of the zinc and acetic acid reduction product of aspergillic acid corresponded to that of tetrahydrodesoxyaspergillic acid, it was thought that desoxyaspergillic acid itself might be reduced to the same product. A

solution of 257 mg. (1.23 mm) of desoxyaspergillie acid in 5.0 ml. of glacial acetic acid was added to a suspension of 50 mg. of reduced platinum oxide catalyst in 1.0 ml. of acetic acid and shaken in an atmosphere of hydrogen. The uptake of hydrogen was quite rapid and became negligible after 1 hour; 2.6 mm, or 2.1 moles per mole of desoxyaspergillie acid, had been absorbed. The catalyst was filtered off and the acetic acid removed *in vacuo*. The residue was dissolved in ether and converted to the hydrochloride with dry HCl. Crystallization from alcohol-ether yielded colorless prisms of melting point 259–260°, and a mixed melting point determination with the product from the zinc reduction of aspergillie acid showed no depression.

*Hydroxypyrazine ( $\alpha$ -Pyrazone, Pyrazinol-2)*<sup>7</sup>—4.75 gm of 2-amino pyrazine were dissolved in 84 ml. of 20 per cent sulfuric acid and treated with a solution of 3.8 gm. of sodium nitrite dissolved in 6 ml. of water. The nitrite solution was added slowly to the vigorously stirred and chilled aminopyrazine solution. After 20 minutes, 24.9 gm. of potassium carbonate were gradually added. The potassium sulfate which separated out was filtered off and the filtrate was evaporated to dryness *in vacuo* at 50°. The residue was digested with absolute ethanol, filtered, and the filtrate treated with norit. On cooling the filtrate, nearly colorless prisms separated out and were filtered off. The yield was 3.2 gm., representing 67 per cent of the theoretical yield. The crystals melted at 183–185°; recrystallized from ethanol, they melted at 185–187°.

*Analysis*— $C_4H_4N_2O$ . Calculated, N 29.16; found, N 29.22

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<sup>7</sup> This preparation of pyrazinol-2 (2-hydroxypyrazine) and 1-methylpyrazinol-2 (1-methyl-2-hydroxypyrazine) from 2-aminopyrazine was carried out in the author's laboratory in March, 1943, by Mr. Jerome N. Haimsohn. The 2-aminopyrazine used as starting material was kindly furnished by Mr. W. A. Lott of the Division of Medicinal Chemistry, the Squibb Institute. The ultraviolet absorption spectrum was reported in September, 1944 (10). Various substituted pyrazinols had previously been reported (23–27), but no ultraviolet absorption characteristics were published. Recently, the synthesis of 3,6-di-*sec*-butylpyrazinol-2 by Newbold and Spring (17) and the synthesis of pyrazinol-2 by Weijlard, Tishler, and Erickson (28) and by Erickson and Spoerri (29) has been reported. The latter workers prepared pyrazinol-2 from 2-aminopyrazine in essentially the same manner as reported here. The melting points and properties of the products are in good agreement. Newbold and Spring (17) state that the ultraviolet absorption spectrum of their synthetic 3,6-di-*sec*-butylpyrazinol-2 is identical with that of desoxyaspergillie acid (Fig. 2). Since none of the other workers has given the absorption characteristics for the pyrazinol-2 derivatives, Fig. 2 is presented, showing the ultraviolet absorption of 2-aminopyrazine, pyrazinol-2, and 1-methylpyrazinol-2. The similarity of these three spectra indicates that pyrazinol-2 and 2-aminopyrazine must exist chiefly in the carbonyl and imino forms.

*1-Methylpyrazinol-2* (*1-Methyl-2-hydroxypyrazine*)—198 mg. of pyrazinol-2 were dissolved in 7 ml. of methanol, added to an ethereal solution of diazomethane which contained approximately 1.2 moles per mole of pyrazinol-2, and allowed to stand in the ice box. After 3 days the ether and excess of diazomethane were evaporated off. The residue was taken up in a small volume of absolute ethanol, treated with norit, filtered, and chilled. Colorless prisms with a melting point of 83–84° were obtained.

*Analysis*— $C_5H_6N_2O$

Calculated. C 54.54, H 5.45, N 25.45

Found. " 54.56, " 5.57, " 25.29,  $OCH_3$  0.0

SUMMARY

Aspergillic acid, an antibiotic substance produced by surface cultures of *Aspergillus flavus*, has been shown to be a pyrazone derivative. The nitrogen atom adjacent to the carbonyl group bears a hydroxyl group and the compound is therefore a cyclic hydroxamic acid. Replacement of the acidic hydroxyl group with hydrogen yields a neutral product called desoxyaspergillic acid. The number and nature of the side chains have been deduced from C-methyl analyses, from the behavior of aspergillic acid towards bromination, and the existence of optical activity.

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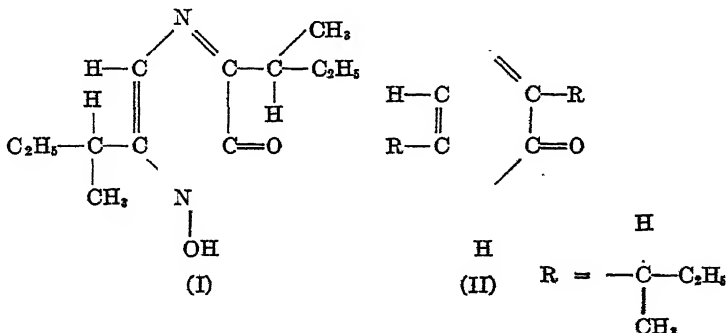
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## II. BROMINATION REACTIONS AND REDUCTION WITH SODIUM AND ALCOHOL

(From the Division of Organic Chemistry, The Squibb Institute for Medical Research,  
New Brunswick)

In Paper I of this series (1) Formula I for the structure of aspergillie acid was deduced from (1) considerations of its chemical properties, (2) the formation of desoxyaspergillie acid (Formula II), and (3) the similarity



Whereas aspergillie acid in glacial acetic acid or chloroform solution is not attacked by bromine, in aqueous solvents it reacts rapidly to form a crystalline product containing 1 atom of bromine. This compound, called bromoaspergillie acid, is very soluble in organic solvents. The crude product melts at low temperature, from 75–80°, but by means of several crystallizations a constant melting point of 129–130° is reached. The analysis showed that 1 hydrogen atom of aspergillie acid had been

replaced by a bromine atom. In methanol solution this compound yielded a deep red color with ferric chloride, and a nicely crystalline green cupric salt was readily obtained by treatment with alcoholic copper acetate solution. These properties showed that the hydroxamic acid function had not been lost. Neither the treatment of aspergillic acid with larger amounts of bromine nor the treatment of bromoaspergillic acid itself with

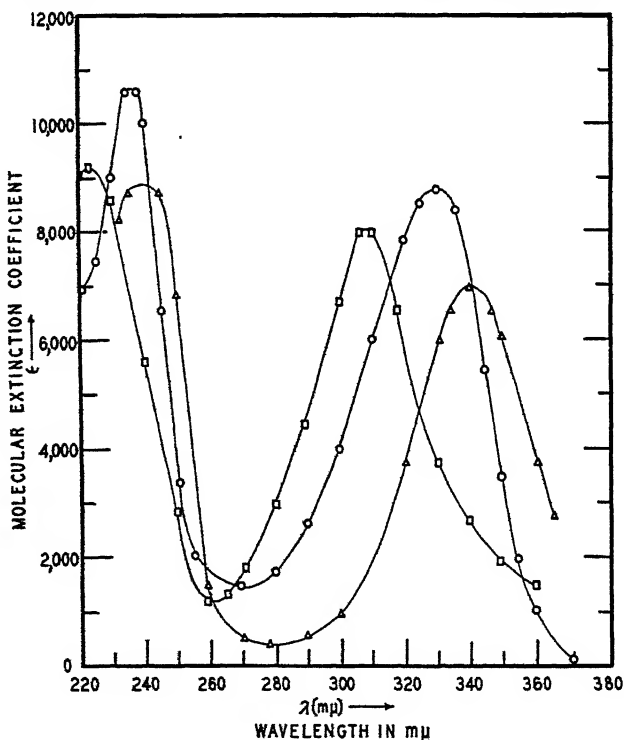


FIG. 1. Ultraviolet absorption spectrum of bromoaspergillic acid ( $\Delta$ ), aspergillic acid (O), bromodesoxyaspergillic acid ( $\square$ ); all determined in ethanol.

more bromine water resulted in the introduction of any more bromine into the molecule.

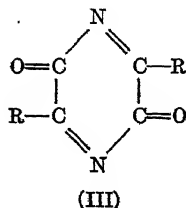
The bromine atom of bromoaspergillic acid proved to be very stably bound. It did not yield silver bromide on treatment with silver nitrate nor could it be hydrolyzed readily with sodium hydroxide solution. The effect of the bromine atom upon the ultraviolet absorption spectrum<sup>1</sup> is shown in Fig. 1. Early tests with less pure material had shown an

<sup>1</sup> The ultraviolet absorption spectra were determined by Dr. Nettie Coy of the Squibb Biological Laboratories, New Brunswick.

increase in antibiotic activity over that of aspergillic acid (2) but subsequent tests with the highly purified bromoaspergillic acid yielded antibiotic values in the same range as aspergillic acid.

In the mother liquors from the purification of bromoaspergillic acid there was present a second compound which contained bromine but was not acidic and did not give the ferric chloride reaction. Analysis showed that this product possessed the formula  $C_{12}H_{13}N_2OBr$  and was thus undoubtedly bromodesoxyaspergillic acid. The preparation of this product from desoxyaspergillic acid directly was therefore investigated. Desoxyaspergillic acid, however, was found to behave somewhat differently from aspergillic acid in that it reacted with bromine in glacial acetic acid to form a crystalline, orange-colored product that was a perbromide. Such perbromide formation is characteristic of heterocyclic bases and especially of pyrazines (3, 4). When the reaction was carried out in aqueous solution, the bromination took place in the same manner as with aspergillic acid. It was thus possible to obtain a product identical with the side product mentioned previously. Again, as with aspergillic acid, the addition of more than 1 mole of bromine did not cause further substitution in the desoxyaspergillic acid.

The yields of bromo products from these reactions are not more than 50 per cent and the nature of the side products is not well established. From the mother liquors of the bromodesoxyaspergillic acid there was obtained a small amount of a colorless high melting product, the analysis of which showed it to be bromine-free and to possess the empirical formula  $C_{12}H_{13}N_2O_2$ . Apparently oxidation had occurred during the bromination. The compound was not acidic nor did it give any ferric chloride reaction, and so could not possess a hydroxamic acid grouping. The 2nd oxygen atom must therefore have been introduced elsewhere than on the nitrogen atom. The low hydrogen number indicated that oxidation of the nucleus had occurred and Formula III was considered a likely structure. Such a structure seemed able also to account for the ultraviolet absorption spectrum as shown in Fig. 2.



The treatment of bromodesoxyaspergillic acid and bromoaspergillic acid with zinc dust in glacial acetic acid solution led in both cases to the



formation of a neutral compound with the empirical formula  $C_{12}H_{22}N_2O_2$ . The course of this reaction, while not immediately clear, must proceed through the hydrolysis of the bromine atom and replacement by a hydroxyl group rather than through reduction and replacement by hydrogen. The reduction of the hydroxyl group on the nitrogen atom in the case of bromo-aspergillic acid was to be expected from the reduction of aspergillic acid

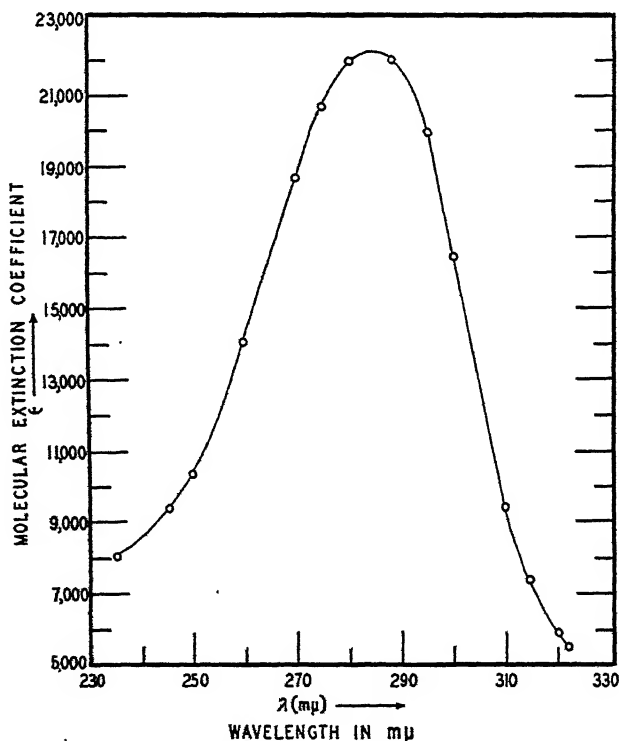


FIG. 2. Ultraviolet absorption spectrum of compound  $C_{12}H_{18}N_2O_2$ ; determined in ethanol.

itself, as reported in Paper I (1). The neutral compound,  $C_{12}H_{22}N_2O_2$ , had a high melting point ( $250^\circ$ ), was optically active, and showed only end-absorption in the ultraviolet region. These properties suggested that it was a diketopiperazine and on the basis of Formula I for aspergillic acid would have the structure shown in Formula IV.

Such a structure represents the diketopiperazine which would be obtained from one of the stereoisomers of isoleucine. The anhydride of natural isoleucine has been reported by Ehrlich (5) who prepared it by dry distillation of L-isoleucine. The properties are listed in Table I for comparison

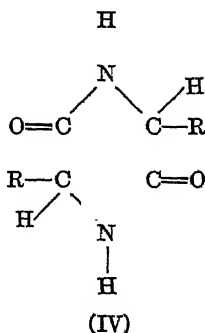
TABLE I  
Comparison of 3,β-Di-sec-butyl-2,5-diketopiperazine from Three Sources

Source	M. p.	$[\alpha]_D$	Crystal form	Solubility
L-Isoleucine*	Sinters at 275°; melts at 280–281°; sublimes at m.p.	Slight dextro-rotation	Clusters of fine needles	Soluble in AcOH, MeOH; less soluble in benzene and acetone
L-Leucine†	270–271°	–42.5°	Longstout prisms	Soluble in AcOH but less so in MeOH and acetone than isoleucine anhydride
DL-Isoleucine‡	Sinters at 247°; melts at 255–257°; sublimes at m.p.	0	Hair-fine needles in clusters from acetone; heavy small prisms from water	Soluble in AcOH, MeOH, less soluble in benzene and acetone; can be crystallized from hot water
Zinc and acetic acid reduction of bromoaspergillic acid	Sinters at 248°; melts at 249–250°; sublimes at m.p.	+13.8°	Hair-fine needles from acetone; heavy small prisms from water	Soluble in AcOH, MeOH; less soluble in benzene, ether, and acetone; can be crystallized from hot water

\* Prepared by Ehrlich (5) by dry distillation of L-isoleucine; because of high temperature used in preparation the product is likely a mixture of isomers.

† Prepared by Fischer (6) and included here for comparison.

‡ Prepared from DL-isoleucine free of alloisoleucine by essentially the same procedure that was used for DL-leucine by Fischer (6).



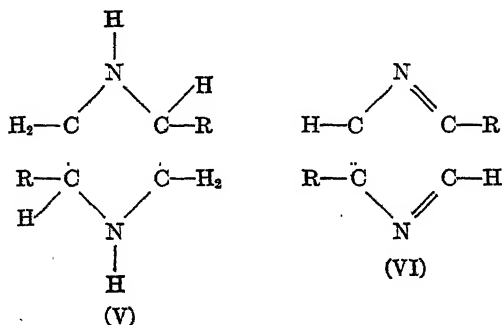
with those of the product from bromoaspergillic acid. A sample of the anhydride of DL-isoleucine was prepared and its properties are also listed

in Table I. While the properties of these compounds are in fair agreement and leave little doubt as to the identity of the product from aspergillic acid, exact comparison is not to be expected because of the mixture of stereoisomers present in the synthetic compounds.

The  $C_{12}H_{18}N_2O_2$  product was reduced catalytically to yield the diketopiperazine of melting point  $250^\circ$ .

As pointed out by Fischer (6) for leucine anhydride and by Ehrlich (5) for isoleucine anhydride, these diketopiperazines are extremely resistant to hydrolysis; therefore the isolation of the constituent amino acid could not be achieved.

To confirm fully the existence of a pyrazine nucleus in aspergillic acid the complete reduction with sodium in amyl alcohol was carried out. From such a reduction an oxygen-free, optically active base was obtained and characterized through the hydrochloride, the picrate, and the dibenzoate. The analyses of these derivatives were in agreement with the formula  $C_{12}H_{20}N_2$  for the base which accordingly should be 2,5-di-sec-butylpiperazine, as shown in Formula V.



Two methods for obtaining a synthetic product for comparison were utilized. The synthetic isoleucine anhydride was reduced with sodium and amyl alcohol to yield the corresponding piperazine (7,8); and 2,5-di-sec-butylpyrazine, Formula VI, which was synthesized in the usual manner from the requisite amino ketone (4), was also reduced with sodium and amyl alcohol. Table II lists the properties of these compounds and their derivatives for comparison. Again, as with the diketopiperazines, exact comparison is rendered difficult by the mixture of stereoisomers represented by the synthetic products. However, it was felt that the comparison achieved by the two series of products was precise enough to make the difficult resolution of such mixtures unnecessary. Since there are eight possible stereoisomers of a piperazine substituted with two other asymmetric groups, the preparation of the individual isomers represents a rather formidable task.

## EXPERIMENTAL

*Bromoaspergillic Acid*—The bromination of aspergillic acid may be carried out with various solvents. Aqueous acetic acid or aqueous ethanol is suitable but strong hydrochloric acid was found to be the most convenient. A solution of 220 mg. of aspergillic acid in 10 ml. of 5 N hydrochloric acid was treated with saturated bromine water until no further

TABLE II  
*Comparison of 2,5-Di-sec-butylpiperazine Derivatives*

	Product from aspergillic acid	Product from DL-isoleucine anhydride	Product from synthetic pyrazine
Picrate	Rhombic plates from ethanol; sinters and darkens at 220–228°; melts at 238–240° with decomposition	Rhombic plates from ethanol; sinters and darkens at 222–225°; melts at 235° with decomposition	Rhombic plates from ethanol; sinters and darkens at 205–225°; melts at 233–235° with decomposition
Hydrochloride	Dense rectangular prisms in clusters; not melted at 300°; $[\alpha]_D = +0.6^\circ$ in $H_2O$		Hexagonal prisms; not melted at 300°
Dinitroso derivative	Nacreous plates; sinters at 120°; melts at 127° with decomposition	Shiny platelets; sinters at 128°; melts at 129–130°	Shiny platelets; sinters at 125–130°; melts at 130–131°
Nitrate	Dense prisms; sinters at 224°; melts at 227° with decomposition	Rectangular prisms; sinters at 225°; melts at 227–228° with decomposition	
Dibenzoate	Needles from ethanol; sinters at 178°; melts at 188–190°; $[\alpha]_D = -7.5^\circ$ in ethanol	Needles from ethanol; sinters at 200°; melts at 203–204°	

precipitate was formed and no more bromine appeared to be consumed. The pale yellow precipitate was filtered off, washed, and dried. It weighed 150 mg. Since this represented only one-half of the expected weight of bromoaspergillic acid, the aqueous filtrate was reserved for further investigation. The crystalline material was very soluble in organic solvents, highly insoluble in water. It dissolved readily in dilute sodium hydroxide or sodium carbonate solution and was reprecipitated by acidification. The alcoholic solution still gave a strong red-brown color with ferric chloride solution. Recrystallization from aqueous methanol or ethanol

did not raise the melting point above 75–80°. However, charcoal treatment of the aqueous alcoholic solution, followed by repeated crystallizations of the product from dry acetone or acetone-pentane mixture, yielded colorless needles with a constant melting point of 129–130°. The rotation in ethanol was  $[\alpha]_D^{25} = +19^\circ$ ,  $c = 1.1$ .

*Analysis*<sup>2</sup>— $C_{12}H_{18}N_2O_2Br$  (303)

Calculated. C 47.50, H 6.27, N 9.24, Br 26.4

Found. " 47.33, " 6.28, " 9.62, " 26.3

The copper salt of bromoaspergillic acid was prepared in the same way as that of aspergillic acid (1). It forms deep green plates from dioxan solution on addition of water. The melting point is 200°, after softening at 195°.

*Analysis*— $(C_{12}H_{18}N_2O_2Br)_2Cu$  (667.6)

Calculated. C 43.10, H 5.39, N 8.38, Cu 9.53

Found. " 43.28, " 5.58, " 8.35, " 8.46\*

\* Determined roughly on the ash.

Bromoaspergillic acid is unchanged by treatment with 0.5 N methanolic KOH at room temperature or by 5 per cent aqueous NaOH at 100° for several hours. No hydrolysis or replacement of bromine by hydroxyl could be achieved by long shaking in alcoholic solution with moist silver oxide.

*Bromodesoxyaspergillic Acid*—The aqueous filtrate after the removal of the precipitated bromoaspergillic acid did not give more precipitate with additional bromine water; on standing in the ice box a crystalline precipitate was slowly deposited, which was collected, washed, and dried. After recrystallization from hot acetone the product melted at 129–130° after softening at 125–126°. A depression of 35° was observed in the mixed melting point determination with bromoaspergillic acid.

*Analysis*— $C_{12}H_{18}N_2OBr$  (287)

Calculated. C 50.10, H 6.62, N 9.75, Br 27.88

Found. " 49.76, " 6.60, " 9.78, " 27.60

*Bromination of Desoxyaspergillic Acid*—Like aspergillic acid, desoxyaspergillic acid reacts readily in aqueous solution with bromine water, but, unlike aspergillic acid, it also reacts with bromine in anhydrous solvents. A glacial acetic acid solution of 366 mg. of desoxyaspergillic acid was treated with a solution of bromine in acetic acid. Decoloration of the bromine did not occur but soon orange-red platelets began to deposit. These were filtered off and recrystallized from glacial acetic acid.

<sup>2</sup> The analyses reported here were carried out by Mr. J. F. Alicino of the Squibb Institute.

After drying in a vacuum desiccator, they weighed 88 mg. In the melting point determination they sintered slightly at 125° and paled to a nearly colorless crystalline mass which decomposed with sublimation at 250–253°. It was apparent that the orange-red platelets represented a perbromide of desoxyaspergillic acid and its composition was determined in the following manner. The material, which had been dried as well as possible in the vacuum desiccator at room temperature, showed a total bromine content of 51.4 per cent and an active bromine content (liberation of iodine from KI) of 30 per cent. These values are in fair agreement with the formulation  $C_{12}H_{20}N_2O \cdot HBr \cdot Br_2$ , which requires total bromine of 53.5 per cent and active bromine 35.6 per cent. When the perbromide is held in the drying pistol at 100° in a vacuum, it is converted to a colorless crystalline salt. The loss in weight by this method agreed with that found for active bromine, 30 to 31 per cent. The colorless crystalline residue agreed in properties with the hydrobromide of desoxyaspergillic acid, m.p. 250–252° with decomposition and sublimation (1).

*Analysis*— $C_{12}H_{20}N_2O \cdot HBr$  (289). Calculated, N 9.68; found, N 9.58

The addition of water to the original filtrate from the bromination of desoxyaspergillic acid caused the immediate discharge of the red color but did not yield a precipitate. The solution was concentrated to dryness *in vacuo* and the residue recrystallized from ethanol. Colorless needles, melting at 125° after softening at 118°, were obtained; they weighed 150 mg. Recrystallization from acetone or aqueous ethanol yielded needles with a melting point of 129–130°.

*Analysis*— $C_{12}H_{19}N_2OBr$  (287). Calculated. C 50.10, H 6.62  
Found. " 50.29, " 6.62

Desoxyaspergillic acid dissolved in N hydrochloric acid or aqueous acetic acid reacted immediately with bromine without the formation of a perbromide. In contrast to bromoaspergillic acid, bromodesoxyaspergillic acid does not precipitate from the solution. To an aqueous acetic acid solution of 1.35 gm. of desoxyaspergillic acid an acetic acid solution of 1.04 gm. of bromine (1 mole per mole) was added. The chilled solution was seeded with crystals of bromodesoxyaspergillic acid and slowly deposited 851 mg. of crude product. From the aqueous filtrate there was obtained by concentration *in vacuo* to a small volume a crop of granular colorless solid which weighed when dry 780 mg. When crystallized from acetic acid, colorless needles melting at 268–270° were obtained. This product proved to be halogen-free.

*Analysis*— $C_{12}H_{18}N_2O_2$  (222). Calculated. C 64.80, H 8.10  
Found. " 64.48, " 8.02

*Zinc and Acetic Acid Reduction of Bromoaspergilllic Acid*—A sample of the bromoaspergilllic acid which is obtained by one recrystallization of the precipitated product and which melts at 75–80° was taken for reduction with zinc dust and acetic acid. To a solution of 378 mg. in 4.0 ml. of glacial acetic acid and 1.0 ml. of water there were added, over a period of 2 to 3 hours refluxing, 5.0 gm. of zinc dust. The hot solution was diluted with 50 ml. of water and allowed to stand in the ice box. There slowly deposited fine colorless needles which were filtered off and dried; weight 103 mg. This compound could be recrystallized from acetone in which it is moderately soluble when hot, but only slightly soluble when cold. It is soluble in methanol and ethanol but only sparingly soluble in benzene or ether. It is slightly soluble in hot water and crystallizes out as clusters of dense prisms on cooling. The melting point is 249–250° with sublimation.

*Synthesis of 3,6-Di-sec-butyl-2,5-diketopiperazine (Isoleucine Anhydride)*—This compound was synthesized in essentially the same manner as reported by Fischer (6) for DL-leucine anhydride except that DL-isoleucine was used as the starting product. 5.17 gm. of DL-isoleucine were converted to the ethyl ester by treatment with dry HCl in 50 ml. of absolute ethanol. After all of the DL-isoleucine had dissolved, the solution was concentrated *in vacuo* at 35° to a syrup from which the free amino ester was liberated by dissolving in ice-cold water and treating with sodium hydroxide solution. The ether extract yielded 3.5 gm. of ester after drying and evaporating at 35°. 3 gm. of the ester were heated in a sealed tube at 200° for 24 hours. The contents of the tube were slightly yellow and partially crystalline. The crystalline material was washed free of unchanged ester with ether and then recrystallized from ethanol. 950 mg. of colorless needles, identical in appearance and solubility characteristics with the natural product (secured by degradation of aspergilllic acid), were obtained. The melting point of the synthetic product was 255–257° with sublimation, after softening at 247°. In a mixed melting point determination with the natural product, there was no lowering of the melting point below 249–250°, which is that of the natural compound.

*2,5-Di-sec-butylpiperazine*—This compound has been obtained by the three following methods.

1. *Sodium and Alcohol Reduction of Aspergilllic Acid*—A solution of 2.25 gm. of aspergilllic acid in 100 ml. of isoamyl alcohol was treated with 10 gm. of metallic sodium added over a period of 1½ hours refluxing. A stream of nitrogen was passed through the apparatus during the reaction. After the solution had been cooled, 100 ml. of water were added and the mixture shaken in a separatory funnel. The amyl alcohol was washed three times with 25 ml. portions of water and then with three 25 ml. portions

of  $N$  HCl. Evaporation of the acidic solution *in vacuo* yielded a crystalline residue which weighed 560 mg. Recrystallization could be achieved from water, in which it is quite soluble, or from methanol by the addition of ether. The crystals separated as heavy prisms or clusters of hexagonal rods; these darkened at  $260^\circ$  but were not melted at  $300^\circ$ . The rotation of a solution of 35.7 mg. in 2 ml. of  $H_2O$  was barely detectable;  $[\alpha]_D =$  about  $+0.6^\circ$ .

*Analysis*— $C_{12}H_{16}N_2 \cdot 2HCl$  (271). Calculated. C 53.10, H 10.32  
Found. " 52.91, " 10.51

An aqueous solution of the hydrochloride on treatment with picric acid solution yielded a precipitate of lemon-yellow tablets. After crystallization from aqueous ethanol, the crystals had a melting point of  $238-240^\circ$  with decomposition; they darkened over the range  $220-228^\circ$ .

*Analysis*— $C_{12}H_{16}N_2 \cdot 2(C_6H_3N_3O_7)$  (656)  
Calculated. C 43.90, H 4.87, N 17.15  
Found. " 44.17, " 5.00, " 17.22

Treatment of an aqueous solution of the base with benzoyl chloride and dilute alkali yielded the dibenzoate which was recrystallized from ethanol. The dibenzoate sinters at  $178^\circ$  and melts at  $188-190^\circ$ .

*Analysis*— $C_{12}H_{14}N_2 \cdot (CO \cdot C_6H_5)_2$  (406). Calculated. C 76.84, H 8.37  
Found. " 76.62, " 8.20

$[\alpha]_D^{24} = -7.5^\circ$  in ethanol,  $c = 3.0$ .

An aqueous solution of the piperazine hydrochloride when treated with dilute  $HNO_3$  slowly deposits clusters of prismatic needles of the nitrate; this salt sinters at  $224^\circ$  and melts with decomposition at  $227^\circ$ .

An aqueous solution of the piperazine hydrochloride when treated with aqueous sodium nitrite solution and then acidified deposited platelets of the dinitroso derivative. Recrystallization from aqueous methanol yielded nacreous plates which softened at  $120^\circ$  and melted with decomposition at  $128^\circ$ .

*Analysis*— $C_{12}H_{24}N_4O_2$  (256). Calculated, N 21.87; found, N 21.60

2. *Sodium and Alcohol Reduction of 3,6-Di-sec-butyl-2,5-diketopiperazine (DL-Isoleucine Anhydride)*—In a similar manner to procedure (1), 500 mg. of the diketopiperazine of melting point  $255-257^\circ$  were treated with 2.5 gm. of elemental sodium added over a 2 hour period. This is essentially the procedure used by Cohn (7) and by Hoyer (8) to obtain the corresponding piperazines from leucine and alanine anhydrides, respectively. The acidic extract was immediately precipitated with picric acid, yielding 275 mg. of the picrate. Crystallization from ethanol yielded rhombic



plates which sintered at 222–225° and melted with decomposition at 235–238°.

*Analysis*— $C_{12}H_{24}N_2 \cdot 2(C_6H_5N_2O_7)$  (656). Calculated. C 43.90, H 4.87  
Found. " 44.19, " 5.08

A portion of the picrate was converted to the free base and from this the dibenzoate, nitrate, and nitroso derivatives were prepared for comparison with the products from the reduction of aspergillic acid (Table II). The inactive dibenzoate had a higher melting point than the product from aspergillic acid but was identical in appearance. It melted at 203–204° after softening at 200°.

*Analysis*— $C_{12}H_{24}N_2 \cdot (CO \cdot C_6H_5)_2$  (406). Calculated. C 76.84, H 8.37  
Found. " 76.56, " 8.16

3. *Total synthesis of this piperazine* was achieved by first preparing 2,5-di-sec-butylpyrazine by a suitable adaptation of the method utilized by Conrad and Hock (4) for the preparation of 2,5-diisopropylpyrazine and then reducing this pyrazine with sodium and alcohol as described by the same authors.

The starting material was ethyl methyl ethyl acetoacetate which was prepared by the customary methods of alkylation (9, 10) and brominated by the procedure of Conrad (11). That fraction of the bromo keto ester which distilled at 165–168° at 78 mm. was treated with alcoholic ammonia solution after it failed to react with aqueous ammonium carbonate solution as described by Conrad and Hock. After the ammonium bromide was filtered off, concentrated HCl was added and the solution heated in a sealed tube at 120° for 5 hours. The resulting solution of amino ketone hydrochloride was concentrated *in vacuo* and, when the volume was small, treated with 33 per cent NaOH and mercuric chloride as described. Steam distillation of this mixture yielded the 2,5-di-sec-butylpyrazine as an oily suspension in the distillate. After extraction with ether, drying over  $Na_2SO_4$ , and evaporation, a clear mobile liquid was obtained which was dissolved directly in isoamyl alcohol and reduced with metallic sodium. The 2,5-di-sec-butylpiperazine was isolated as the hydrochloride, as described under procedure (1), and characterized as the picrate and dinitroso derivatives. The appearance and melting points of these products were identical with those previously obtained and are listed in Table II.

#### SUMMARY

The structure deduced for aspergillic acid in Paper I (1), and shown in Formula I, has been substantiated by a series of degradations including (1) bromination to bromoaspergillic acid, (2) reaction of this with zinc in acetic acid to yield 3,6-di-sec-butyl-2,5-diketopiperazine, and (3) reduc-

tion of aspergillie acid with sodium and isoamyl alcohol to yield 2,5-di-sec-butylpiperazine. The identification of these degradation products has been achieved by comparison with the synthetic compounds.

The stimulating encouragement of this work by Dr. Oskar Wintersteiner is gratefully acknowledged.

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## FURTHER STUDIES ON THE AMINO ACID COMPOSITION OF IMMUNE PROTEINS

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Although the protein nature of antibodies is now firmly established, there is little available information concerning their amino acid composition. Studies recently reported from this laboratory have provided methods of isolation which have made available sufficient amounts of immune proteins for analytical study (1-3). These proteins are for the most part electrophoretically homogeneous, but are not completely homogeneous in the ultracentrifuge (2). Moreover, the limitation should be recognized that these proteins were all isolated by physicochemical methods, and not by immunologically specific precipitation. Each protein fraction, therefore, includes a considerable portion of material which is not immunologically specific.

Brand, Kassell, and Saidel (4) and Brand (5) have already reported analyses on a  $\gamma$ -globulin preparation from human plasma. A previous paper from this laboratory (Smith, Greene, and Bartner (6)) has dealt with the content of carbohydrate and of several amino acids of the immune proteins which have been isolated from human, horse, and bovine plasma, and with the immune lactoglobulins of the cow. The present paper presents analyses on this group of proteins for seven additional amino acids.

### EXPERIMENTAL

The proteins used in this investigation were all prepared in this laboratory, and, with the exception of the milk globulins, are the same preparations as those already analyzed for carbohydrate and other amino acids (6). The isolation and some properties of the bovine colostrum and plasma proteins have been described by Smith (1), and of the milk proteins by Smith (2). Preparation A of the  $\gamma$ -globulin was derived from normal steer blood obtained at the slaughter-house; Preparation B was isolated from the plasma of cows hyperimmunized with mixed antigens, and the colostrum, milk, and T-globulins were from the same hyperimmune animals.

The horse  $\gamma$ - and T-globulins were isolated by alcohol precipitation methods from plasma containing tetanus antitoxin, and these globulins were high in antitoxic protection for guinea pigs. The isolation of these

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proteins has been reported by Smith and Gerlough (3). The human  $\gamma$ -globulin preparations were electrophoretically homogeneous and were prepared in this laboratory by Method 9 of Oncley and his collaborators (7).

Microbiological assays for arginine, histidine, lysine, isoleucine, threonine, and methionine were performed with *Streptococcus faecalis* following the procedure described by Stokes, Gunness, Dwyer, and Caswell (8). The growth of the bacteria was followed by titration of the lactic acid produced. Hydrolysis of 200 to 500 mg. of protein in 10 ml. of 3 N HCl was performed

TABLE I

*Arginine, Histidine, and Lysine Content of Immune Proteins*

The analytical data are given for the anhydrous ash-free proteins. The averages and the average deviations are based on determinations of at least four separate hydrolysates.

Protein	Arginine	Histidine	Lysine	Total basic amino acids
	per cent	per cent	per cent	moles $\times 10^{-5}$ per gm. protein
Colostrum euglobulin.....	5.06 $\pm$ 0.08	1.83 $\pm$ 0.11	6.2 $\pm$ 0.1	83
Milk euglobulin.....	4.76 $\pm$ 0.10	1.95 $\pm$ 0.15	6.3 $\pm$ 0.1	83
Colostrum pseudoglobulin....	3.64 $\pm$ 0.37	2.13 $\pm$ 0.17	7.2 $\pm$ 0.3	84
Milk pseudoglobulin.....	3.34 $\pm$ 0.23	2.14 $\pm$ 0.06	7.2 $\pm$ 0.1	82
Colostrum total globulin A....	4.36 $\pm$ 0.04	1.99 $\pm$ 0.16	6.3 $\pm$ 0.1	81
"    "    "    B....	4.67 $\pm$ 0.20	1.84 $\pm$ 0.12	6.4 $\pm$ 0.1	82.5
Bovine $\gamma$ -globulin A.....	6.02 $\pm$ 0.15	2.01 $\pm$ 0.17	6.8 $\pm$ 0.2	94
"    "    B.....	5.60 $\pm$ 0.15	2.09 $\pm$ 0.18	6.6 $\pm$ 0.2	91
Bovine T-globulin.....	4.79 $\pm$ 0.12	2.01 $\pm$ 0.03	6.4 $\pm$ 0.1	84
Horse $\gamma$ -globulin.....	3.77 $\pm$ 0.17	2.44 $\pm$ 0.14	8.6 $\pm$ 0.2	96
"    T-globulin.....	2.80 $\pm$ 0.22	2.43 $\pm$ 0.09	6.7 $\pm$ 0.2	78
Human $\gamma$ -globulin II-1,2	5.12 $\pm$ 0.28	2.01 $\pm$ 0.19	7.2 $\pm$ 0.3	92
"    "    II-3.....	3.66 $\pm$ 0.26	1.91 $\pm$ 0.10	6.3 $\pm$ 0.2	77

in the autoclave in sealed ampules for 7 hours. After hydrolysis, the neutralized solutions were assayed by comparison with standard amino acid growth tubes. For each hydrolysate, the average of several measurements at different growth levels was considered as a single determination. The assays were controlled by parallel determinations on a 3 times crystallized preparation of  $\beta$ -lactoglobulin.<sup>1</sup>

*Arginine, Histidine, and Lysine Determinations*—The microbiological determinations of the basic amino acids in these proteins are presented in Table I. Brand (5) has reported for a preparation of human  $\gamma$ -globulin

<sup>1</sup> The technical assistance of Lois Herbert, Rachel Mato, and Helen Zeveney in the microbiological assays is gratefully acknowledged.

II-1 4.80 per cent arginine, 2.50 per cent histidine, and 8.1 per cent lysine. His values for the last two amino acids are somewhat higher than those obtained by us. Vickery (9) found 2.66 per cent arginine in a  $\gamma$ -pseudoglobulin from normal horse serum isolated by Green. This value agrees with our finding of 2.80 per cent arginine in T-globulin rather than with that of 3.77 per cent in our preparation of horse  $\gamma$ -globulin.

The acid-combining capacity of proteins is determined by the sum of the basic amino acids and of the free  $\alpha$ -amino groups. Although data are lacking for the latter, we have computed the total content of basic amino

TABLE II  
*Isoleucine and Threonine Content of Immune Proteins*

The analytical data are for the ash-free and moisture-free proteins. The average values and the average deviations are based on four separate hydrolysates. The isoleucine data are in terms of a standard known to be free of the allo compound.

Protein	Isoleucine	Threonine
	<i>per cent</i>	<i>per cent</i>
Colostrum euglobulin.....	3.14 $\pm$ 0.18	10.3 $\pm$ 0.3
Milk euglobulin.....	3.02 $\pm$ 0.01	10.6 $\pm$ 0.6
Colostrum pseudoglobulin.....	3.23 $\pm$ 0.13	9.8 $\pm$ 0.2
Milk pseudoglobulin.....	3.04 $\pm$ 0.12	10.3 $\pm$ 0.3
Colostrum total globulin A.....	2.77 $\pm$ 0.11	9.4 $\pm$ 0.1
"    "    B.....	3.00 $\pm$ 0.09	10.1 $\pm$ 0.7
Bovine $\gamma$ -globulin A.....	3.05 $\pm$ 0.13	9.8 $\pm$ 0.2
"    "    B.....	3.31 $\pm$ 0.17	10.1 $\pm$ 0.7
"    T-globulin.....	3.00 $\pm$ 0.09	9.5 $\pm$ 0.4
Horse $\gamma$ -globulin.....	4.39 $\pm$ 0.02	11.1 $\pm$ 0.4
"    T-globulin.....	3.26 $\pm$ 0.08	8.7 $\pm$ 0.3
Human $\gamma$ -globulin II-1, 2.....	2.80 $\pm$ 0.23	8.8 $\pm$ 0.4
"    "    II-3.....	2.00 $\pm$ 0.06	7.4 $\pm$ 0.5

acids (Table I) in order to facilitate comparison of these proteins with the available data on their acid-combining capacity. Perlmann (10) found for horse  $\gamma$ -pseudoglobulin  $89 \times 10^{-5}$  moles per gm. of protein by measuring the bound metaphosphoric acid, and she cites the unpublished titration data of Cohn, Green, and Blanchard who found  $92 \times 10^{-5}$  moles per gm. of protein. Green (11) has studied the acid-combining capacity of several horse globulins, and has reported values of 100 for globulins PI and PII, and 90 for globulin PIII. These values are all in reasonable agreement with our analytical value for horse  $\gamma$ -globulin of  $96 \times 10^{-5}$  moles per gm. of protein.

The  $\gamma$ -globulins of cow and horse plasma have a higher content of basic amino acids, and, therefore, probably a higher acid-combining

capacity than the corresponding T-globulins of these species. This is in accord with the more alkaline isoelectric points of the  $\gamma$ -globulins. In fact, for all of the immune proteins there is general agreement with the idea that the more alkaline the isoelectric point, the higher the content of basic amino acids. The lower content of basic amino acids, and the more acid isoelectric point of the human  $\gamma$ -globulin II-3 emphasize its resemblance to the T-globulins of the horse and the cow.

TABLE III

*Cystine and Methionine Content of Immune Proteins*

The data are for the anhydrous ash-free proteins. The methionine content estimated microbiologically is based on four separate hydrolysates. The cystine content of these proteins is the average of two or three determinations. The calculated sulfur values are obtained from the sum of the cystine and methionine contents.

Protein	Cystine	Methionine	Sulfur calculated	Sulfur found
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Colostrum euglobulin.....	3.14	$1.10 \pm 0.02$	1.11	1.09
Milk euglobulin.....	3.28	$0.87 \pm 0.05$	1.03	1.01
Colostrum pseudoglobulin.....	3.04	$1.23 \pm 0.02$	1.08	1.08
Milk pseudoglobulin.....	3.02	$0.92 \pm 0.07$	1.00	1.00
Colostrum total globulin A.....	3.26	$0.89 \pm 0.07$	1.06	1.01
"    "    "    B.....		$0.92 \pm 0.04$		
Bovine $\gamma$ -globulin A.....	2.89	$1.14 \pm 0.03$	1.02	1.05
"    "    B.....	2.95	$1.22 \pm 0.05$	1.05	0.99
"    T-globulin.....	2.80	$1.00 \pm 0.02$	0.96	0.95
Horse $\gamma$ -globulin.....	2.57	$0.95 \pm 0.06$	0.89	1.00
"    T-globulin.....	2.52	$0.72 \pm 0.03$	0.83	0.91
Human $\gamma$ -globulin II-1,2.....	2.55	$1.12 \pm 0.11$	0.92	1.00
"    "    II-3.....	2.71	$0.87 \pm 0.03$	0.91	0.99

*Isoleucine and Threonine*—In Table II are given the analytical data for these amino acids as obtained by microbiological assay. Brand (5) has reported 2.7 per cent isoleucine and 8.4 per cent threonine for human  $\gamma$ -globulin II-1, results which are very close to the values obtained for the preparation of  $\gamma$ -globulin II-1,2 analyzed by us. The relatively high content of threonine in all of the immune proteins is noteworthy.

*Cystine and Methionine Content*—Table III presents the data for the sulfur-containing amino acids of the immune proteins. The methionine estimations were performed microbiologically as described above. The cystine (cystine + cysteine) content of these proteins was determined by the procedure of Kassell and Brand (12).<sup>2</sup> The total sulfur of these

<sup>2</sup> We are indebted to Mr. Elliot Bartner for these determinations.

proteins has been calculated from the estimations of cystine and methionine, and compared with the sulfur content as found by microanalysis. The sulfur determinations have been published in earlier papers on these proteins (1, 2, 6). New determinations of the sulfur content of the horse T-globulin indicate that the sulfur content of this protein is 0.91 per cent rather than the lower value of 0.69 per cent which was reported earlier (6).<sup>3</sup>

It is apparent from the data in Table III that the cystine and methionine content of the bovine proteins satisfactorily account for their sulfur content within the experimental error of the several determinations. However, some discrepancies are apparent in the analyses of the horse and human proteins. Brand, Kassell, and Saidel (4) found 1.06 per cent methionine and 3.07 per cent cysteine + cystine in human  $\gamma$ -globulin II-1.

#### DISCUSSION

The most striking fact concerning the immune proteins which we have analyzed is their great similarity in composition. Although many significant differences can be observed between them, these proteins form a fundamental group with very similar properties. The immune proteins of plasma are different from the other plasma proteins which have been analyzed. Brand, Kassell, and Saidel have already shown this by comparative analyses of various human proteins. The immune globulin preparations from milk and colostrum show amino acid compositions quite distinct from those of the other well characterized milk proteins, casein and  $\beta$ -lactoglobulin. Thus, the immune proteins clearly show by their composition their similar function, and do not reflect the particular fluid, plasma, milk, or colostrum, from which they are derived.

It is particularly worthy of emphasis that the two bovine  $\gamma$ -globulins, one from normal and the other from hyperimmune animals, do not show any differences which are greater than the errors of analysis. Since our results in this paper and in an earlier one (6) now include eleven amino acids, as well as hexose and hexosamine, it is probably safe to assume that no great differences between the two  $\gamma$ -globulin preparations will be found.

<sup>3</sup> The value for the sulfur content of the horse T-globulin was first reported as 0.69 (1, 2, 6). After the amino acid analyses were made, it was apparent that a considerable discrepancy existed between the sulfur value calculated from the observed cystine and methionine content and that obtained by direct analysis. New analyses by the micro-Pregl method gave 0.93, 0.87, and 0.94 per cent for an average of 0.91 per cent. The newer results emphasize the resemblance between the horse T-globulin and the other immune proteins, and lessen the importance of the sulfur value in distinguishing these proteins (3, 6). The authors are indebted to Mr. J. F. Alicino of The Squibb Institute for Medical Research for the sulfur determinations.



An extension of this series to include other amino acids, and additional proteins from so called normal animals for comparison with those obtained from hyperimmune ones, might prove to be of value in understanding the specific nature of antibodies. Results obtained by other investigators in less extensive analyses have also failed to establish differences between antibodies and normal globulin (13-17). It must be reemphasized (Smith and Coy (18)), however, that such comparisons must be performed on the same globulin fraction from normal and hyperimmune animals, since different globulin fractions concerned with immunity do not possess the same amino acid composition.

The various bovine immune proteins show significant differences in composition from each other (6). The data included in this paper reinforce the conclusion that the immune proteins present in the milk and colostrum are different from those in the plasma. In particular,  $\gamma$ -globulin is strikingly different in arginine content from the T-globulin or the lactoglobulins. The corresponding lactoglobulins from milk and colostrum are very similar in composition to each other. The minor differences probably reflect the fact that none of the proteins is completely homogeneous in the ultracentrifuge, and that it is impossible to be certain of thorough separation of the euglobulin and pseudoglobulin components.

The  $\gamma$ - and T-globulins of the horse differ quite markedly from each other in arginine, lysine, isoleucine, threonine, methionine, and, as previously demonstrated (6), in leucine content.

The two human protein fractions differ considerably in their composition. In most characteristics, the  $\gamma$ -globulin II-3 fraction resembles the T-globulins of the horse and the cow. In this connection, we can point out the more acid isoelectric point of the II-3 fraction (7), its electrophoretic mobility which is intermediate between those of the  $\gamma$ -globulin II-1,2 and the  $\beta$ -globulins (7, 19), its lower content of basic amino acids, isoleucine, threonine, and methionine. It is probable that this fraction (II-3) is completely analogous to the T-globulin of other species. This has been pointed out recently by Deutsch, Alberty, and Gosting (19) on the basis of the electrophoretic mobility of this fraction. The  $\gamma_1$ - and  $\gamma_2$ -globulins of these investigators are apparently identical with the  $\gamma$ -globulins II-3 and II-1,2 of Oncley *et al.* (7).

Evidence has recently been reported that the  $\gamma$ - and T-globulins do not include all of the antitoxic activity present in the plasma of the horse (3). It has also been found that the  $\gamma$ - and T-globulins of the cow do not possess all of the antibody activity found in the plasma of this species.<sup>4</sup> Since these additional immune proteins have not yet been isolated in relatively

<sup>4</sup> Unpublished observations by one of us (E. L. S.).

homogeneous form, no analyses are available. However, it may safely be assumed that these additional immune proteins will be found to be different in composition from the  $\gamma$ - and T-globulins, since it is already known that they are different in electrophoretic mobility and in their isoelectric points.

The composition of the immune proteins has particular significance for the economy of the organism in its ability to organize resistance to infection. The synthesis of the globulins concerned with immunity is then a special problem in nutrition, as emphasized recently by Cannon (20). The data obtained in our investigations show that all of the amino acids known to be essential for the maintenance of nitrogen equilibrium in man and other mammals are present in the immune proteins of the three species which we have studied. This fact alone indicates the necessity for a complete supply of the essential amino acids for the synthesis of immune proteins. In addition, strong evidence is available which shows that hypoproteinemia causes a decreased synthesis of antibodies and a lowered resistance to infection (20).

#### SUMMARY

1. The immune proteins isolated from the plasma of human, horse, and cow and the lactoglobulins from the colostrum and milk of the cow have been analyzed for arginine, histidine, lysine, isoleucine, threonine, and methionine by microbiological assay with *Streptococcus faecalis*. Cystine analyses have been performed by the photometric method of Brand and Kassell.

2. For the plasma proteins, the  $\gamma$ -globulins of the horse and cow have a higher content of basic amino acids, isoleucine, threonine, and methionine, than the T-globulins of the same species. For the human proteins, the  $\gamma$ -globulin II-1,2 resembles the  $\gamma$ -globulins of the other species, and the  $\gamma$ -globulin II-3 is similar to the T-globulins.

3. The entire group of immune proteins is very similar in composition. The high content of threonine (7.4 to 11.1 per cent) is particularly noteworthy. The two sulfur-containing amino acids, cystine and methionine, account reasonably well for the sulfur content of these proteins.

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# ON THE RÔLE OF THE OXIDATION IN THE METHYLATION OF GUANIDOACETIC ACID\*

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There are two, at least, methyl transfer reactions promoted by liver slices *in vitro* (2). The fundamental distinction between them is that one is dependent on oxygen and the other is not.

Transmethylation reactions found to be independent of oxygen are methionine formation by the methylation of homocysteine, homocystine, or homocysteine thiolactone by either choline or betaine. They are not inhibited by oxidation inhibitors. Homogenized tissue and slices are equally effective without any modification of the reaction mixture.

Transmethylations found to be dependent on oxygen are the methylation of guanidoacetic acid to form creatine and of nicotinamide to N<sup>1</sup>-methylnicotinamide (3). The methyl donor is methionine; choline and betaine are ineffective. Neither reaction in this category proceeds when the tissue (liver) is homogenized or finely minced and the reaction mixture consists only of methyl donor and acceptor.

After many unsuccessful experiments to gain further insight into the rôle of the oxidation in the methylation of guanidoacetic acid with rat liver slices we turned to the use of liver homogenates. We found that guanidoacetic acid is methylated by methionine in rat and guinea pig liver homogenates if sufficient adenosine triphosphate (ATP) is provided. The yields with rat liver homogenate were low and irregular. Guinea pig liver homogenates were better; they gave, on the average, more than twice the creatine obtained with rat liver slices (on the basis of dry weight of tissue used), and the results were consistent. The general picture of the reaction was the same as with rat liver slices. Accordingly we have confined ourselves in the study of the rôle of the oxidation in the methylation of guanidoacetic acid to homogenates of the livers of guinea pigs.

## Methods

In the experiments with liver slices the animals used were adult white rats of both sexes which had been bred from the Wistar Institute strain.

\* This work is a part of that done under contract with the Office of Naval Research, United States Navy Department. Presented at the meeting of the American Society of Biological Chemists, May 18-24, 1947 (1).

The homogenates were made from the livers of guinea pigs of both sexes, obtained commercially as needed.

The animals were killed by stunning and bled thoroughly. The technique, when liver slices were used, has been described in a previous communication (2).

For the preparation of the homogenates the liver was chilled in ice water immediately after removal, washed free of blood by ice-cold buffer solution, cut into small pieces, and then homogenized in the apparatus of Potter and Elvehjem (4) in a volume of ice-cold buffer solution equal to twice the weight of the tissue. The homogenization apparatus was jacketed in an ice and water bath. At the end of the homogenization the temperature of the homogenate had risen to 4°. In order to obtain a fine homogenate it was prepared in two stages, first in a tube in which the clearance of the rotor was 0.4 mm., followed, after straining through two layers of cheese-cloth, by a second homogenization with a rotor clearance of 0.1 mm.

The buffer solution was made according to the recipe of Cohen and Hayano (5) at pH 7.4.

The reaction vessels were 20 ml. beakers. They were contained in a specially designed heat-regulated, gas-equilibrated reaction chamber.<sup>1</sup>

The final volume of the reaction mixtures (including that of the homogenate) was, in any one experiment, 3.0, 3.5, or 4.0 ml., whichever was convenient. The reaction mixtures were made up before the animal was killed and set away in an ice bath; the homogenate was pipetted into each as soon as it was prepared. The usual time from the killing of the animal to the beginning of the incubation at 38° was about 20 minutes. The contents of the reaction vessels came to the temperature of the bath in about 5 minutes. During this interval the oxygen or nitrogen was passed through in a vigorous stream, after which it was slowed down to a slight positive pressure which was maintained throughout the experimental period. From the time they were set in the water bath to the end of the experimental period the reaction vessels were rocked at a rate of 80 cycles per minute.

At the end of an experimental run the reaction was stopped by adding to each reaction vessel 2 drops of 1 N HCl. Water was then added to a desired volume, the pH adjusted to 5.0, and the vessels immersed in a boiling water bath for 5 minutes, after which they were cooled to room temperature and the contents filtered.

3 ml. aliquots were taken for analysis. 1 ml. of 0.4 N HCl was added to each; they were then autoclaved at 20 pounds pressure for  $\frac{1}{2}$  hour, cooled, the creatinine adsorbed on Lloyd's reagent, the clay washed twice with 3

<sup>1</sup> This apparatus was described briefly in a previous communication (2). A detailed description will be submitted for publication shortly.

ml. of 0.01 N HCl, and the creatinine assayed with alkaline picrate by the method previously described (6). Creatine standards were carried through the whole analytical procedure simultaneously with the experimental samples. The range of the standards and unknowns corresponded. A Klett-Summerson photoelectric colorimeter, with the green filter, was used.<sup>2</sup>

### Reagents

The following were the substances used and their sources: guanidoacetic acid prepared by the method of Nencki and Sieber (11); N-phosphoguanidoacetic acid prepared by the method of Fawaz and Seraidarian (12); two preparations of L-methionine, one kindly donated by Professor W. C. Rose and one prepared by ourselves (2); D-methionine, also donated by Professor Rose; DL-methionine obtained from two commercial sources; L-methionine sulfoxide and L-methionine sulfone prepared by the methods of Toennies and Kolb (13, 14); L-dehydromethionine prepared by the method of Lavine (15);  $\alpha$ -ketomethiol butyrate prepared by the method of Cahill and Rudolph (16);  $\alpha$ -ketoglutaric acid prepared by the method of Schneider (17); fumaric acid purified by four recrystallizations from a commercial preparation; ATP prepared by the method of LePage (18); adenylic acid obtained from two commercial sources; cytochrome c prepared by the method of Keilin and Hartree (19); the oxidation inhibitors were commercial C.P. preparations.

<sup>2</sup> The alkaline picrate method is not specific for creatinine (7). Nevertheless we have used it for the following reasons. Determination of true creatine (as creatinine) by the difference in color with alkaline picrate before and after digestion with the specific bacterial preparation of Miller *et al.* (7, 8) is too cumbersome and time-consuming for experiments in which there are a large number of control and experimental solutions. And the number of variables which can be tested in any one experiment is greatly reduced because of the necessary duplication both of the analyses and of the number of samples of each experimental variable under test. Most of the non-creatinine chromogenic material (except glycohydrazide) is removed by adsorption on Lloyd's reagent and subsequent washing of the clay. In an earlier study (9) we found that deducting the color given by tissue alone plus that given by guanidoacetic acid alone gave, without prior bacterial digestion, values for creatine formed  $90 \pm 5$  per cent of those obtained by difference before and after bacterial digestion. In this study we were interested only in the amount of creatine formed from guanidoacetic acid and not in absolute values. As will be seen below, with the method used (*i.e.* without bacterial digestion and deducting the tissue and guanidoacetic acid blank values) no creatine formation was noted when either guanidoacetic acid or methionine was withheld from the reaction mixture. The rate of creatine formation was greater the higher the initial concentration of either guanidoacetic acid or methionine. We consider, therefore, that the method used gave a true picture of creatine formation in the experiments described below. Handler and Bernheim (10) came to a similar conclusion.

When nitrogen was used for anaerobic experiments, the residual oxygen was removed by passing it over red-hot copper filings in a muffle furnace.

### Results

Table I is a summarized protocol of a typical experiment showing the formation of creatine from guanidoacetic acid in guinea pig liver homogenate. The figures show the necessity of methionine and a doubling of

TABLE I

*Methylation of Guanidoacetic Acid to Creatine in Guinea Pig Liver Homogenate; Effect of Added ATP and of Methionine*

Volume, 3 ml. 1 ml. of homogenate consisting of 1 part of liver and 2 parts of buffer. Initial concentrations of reactants, guanidoacetic acid  $2.5 \times 10^{-4}$  M, L-methionine  $1.5 \times 10^{-3}$  M, ATP (purity 93 per cent)  $1.5 \times 10^{-3}$  M. The pH of all solutions was adjusted to 7.4. The experiment was run for 1 hour at 38° under oxygen. The figures given are the averages of triplicates.

The gross "creatine" (Column 5) represents the total color developed in alkaline picrate compared with that of the creatine standards. The formed creatine (Column 6) represents (gross "creatine") - (tissue gross "creatine" + guanidoacetic acid gross "creatine"). In computing the figures in Column 7 it was assumed that the liver consists of 20 per cent solids. The dry weight of liver in each reaction vessel was on this basis  $0.33 \times 0.2$  gm. = 66 mg. The slightly higher tissue blank with added ATP was due to color in the ATP preparation used.

Tissue	Guanidoacetic acid	Methionine	ATP	Gross "creatine" in filtrate	Formed creatine in filtrate	Formed creatine per gm. (dry weight) liver per hr.
(1)	(2)	(3)	(4)	(5)	(6)	(7)
				mg. per cent	mg. per cent	micromoles
+	-	-	-	0.70		
+	+	-	-	0.84	0	0
+	-	+	+	0.74		
+	+	+	-	1.09	0.25	2.3
+	+	-	+	0.88	0	0
+	+	+	+	1.44	0.56	5.2
-	+	-	-	0.14		

the yield by the addition of ATP with methionine. There was no methylation of guanidoacetic acid by the homogenate alone or when ATP was added without methionine.

The methylation of guanidoacetic acid by methionine when no ATP was added we have interpreted as due to ATP present in the homogenate or formed during the incubation. This interpretation is supported by the findings reported below, and by analogous findings on hippuric acid (20) and urea (21) synthesis in guinea pig liver homogenate. The latter reactions proceed without added ATP and are accelerated when it is added.

The augmenting effect of ATP varied in different homogenates. In eight other experiments the ratios of the amount of creatine formed with added ATP to the amount without ATP were 8.5, 6.6, 2.7, 2.2, 2.0, 1.9, 1.9, and 1.8.

The yield of creatine in guinea pig liver homogenate when ATP was added to the reaction mixture was, on the average, approximately double that given by rat liver slices. 5.2 micromoles per gm. (dry weight) of guinea pig liver per hour (Table I) was typical. In rat liver slices the yield on the same basis varied from 1.0 to 5.0 micromoles (9), with 2.5 to 3.0 micromoles as the median range.

The inferences from results such as those in Table I were strong that one function (if not the exclusive function) of the oxidation in the methylation of guanidoacetic acid by methionine in liver slices was the continuous provision of sufficient ATP, that the amount of the latter initially present in the slices was either insufficient or that it quickly disappeared, and that ATP produced in the slices as a concomitant of oxidations was available for the transmethylation reaction. It would support this interpretation if it were found in guinea pig liver homogenate that ATP produced during the incubation gave results similar to those by ATP added to the reaction mixture initially. Ochoa (22) showed that oxidation of  $\alpha$ -ketoglutarate is obligatorily coupled with the esterification of inorganic phosphate and that in the process ATP is formed. Kalckar (23) found that the oxidation of malate or fumarate is associated with an intense phosphorylation; ATP formation was not demonstrated directly, but its intermediate formation could be inferred. Accordingly in a number of experiments adenylic acid plus either  $\alpha$ -ketoglutarate or fumarate was added to the reaction mixture instead of ATP.

A typical set of results is given in Table II. They show that adenylic acid plus either  $\alpha$ -ketoglutarate or fumarate was somewhat more effective than an initial addition of ATP, and as effective as ATP plus one of the acids. There was no additive effect of  $\alpha$ -ketoglutarate and fumarate in the concentrations (0.01 M) used.

Results such as those in Table II strengthen the conclusion that ATP is required for the transmethylation and that one of the functions of oxygen both in slices and in homogenates is to support oxidations which provide ATP.

The interpretation we have put on the higher yield of creatine when either  $\alpha$ -ketoglutarate or fumarate was added initially with ATP is that ATP diminishes in the homogenate during the incubation, whether by phosphatase action or by being used in reactions in addition to that of transmethylation. Hence when provision is made for its reconstitution by the addition and oxidation of such metabolites as  $\alpha$ -ketoglutarate or fuma-



rate, a higher rate of transmethyations was maintained. This interpretation accounts, qualitatively, also for the somewhat higher yield of creatine with adenylic acid plus either  $\alpha$ -ketoglutarate or fumarate than with ATP alone.

The addition of cytochrome *c* to the reaction mixture, over a concentration range of  $10 \times 10^{-6}$  to  $4 \times 10^{-6}$  M did not increase the yield of creatine.

TABLE II

*Methylation of Guanidoacetic Acid to Creatine in Guinea Pig Liver Homogenate; Effect of ATP Formed during Reaction*

Volume, 3.5 ml. 1 ml. of homogenate consisting of 1 part of liver and 2 parts of buffer. Initial concentrations of reactants, guanidoacetic acid  $8 \times 10^{-4}$  M, L-methionine  $1 \times 10^{-2}$  M, ATP (purity 93 per cent)  $1.5 \times 10^{-3}$  M, adenylic acid  $1 \times 10^{-3}$  M,  $\alpha$ -ketoglutarate  $1 \times 10^{-2}$  M, fumarate  $1 \times 10^{-2}$  M. The pH of all solutions was adjusted to 7.4. The experiment was run for 1 hour at  $38^\circ$  under oxygen.

The figures given are the averages of triplicates.

The values in Columns 9 and 10 were computed on the same basis as the corresponding values in Table I.

Tissue	Guanidoacetic acid	Methionine	ATP	Adenylic acid	$\alpha$ -Keto-glutaric acid	Fumarate	Gross "creatine" in filtrate	Formed creatine in filtrate	Formed creatine per gm. (dry weight) liver per hr.
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
							mg. per cent	mg. per cent	micromoles
+	-	+	-	+	+	+	0.72		
+	+	-	-	+	+	+	1.13	0	0
+	+	+	-	+	-	-	1.19	0.06	0.6
+	+	+	-	+	+	-	1.74	0.61	6.0
+	+	+	-	+	-	+	1.73	0.60	5.9
+	+	+	-	+	+	+	1.73	0.60	5.9
+	-	+	+	-	+	+	0.78		
+	+	-	+	-	+	+	1.19	0	0
+	+	+	+	-	-	-	1.70	0.51	5.0
+	+	+	+	-	+	-	1.83	0.64	6.3
+	+	+	+	-	-	+	1.86	0.67	6.6
+	+	+	+	-	+	+	1.80	0.61	6.0
-	+	-	-	-	-	-	0.41		

This was found in both rat and guinea pig liver homogenates, whether or not ATP,  $\alpha$ -ketoglutarate, or fumarate was added. The concentration of cytochrome *c* is evidently not a limiting factor in the homogenates used.

We have explored, cursorily, the effect of the initial concentrations of the catalyst and of the major reactants on the rate of creatine formation. A more thorough study has been postponed until a purified enzyme preparation is available. Briefly the findings were as follows: The same amount of creatine was formed in 1 hour at  $38^\circ$  with 0.2 and 0.1 gm. of fresh liver

per ml. of reaction mixture; half that amount was obtained with 0.05 gm. and one-eighth with 0.025 gm. of liver per ml. of reaction mixture.

Tripling the initial concentration of guanidoacetic acid from  $0.26 \times 10^{-3}$  to  $0.78 \times 10^{-3}$  M increased the rate 50 per cent. Increasing the initial methionine concentration from  $1 \times 10^{-3}$  to  $1 \times 10^{-2}$  M also increased the rate 50 per cent.

The effects of increasing the initial concentrations of both guanidoacetic acid and of methionine were additive. The following is a typical result. With initial concentrations of guanidoacetic acid and of methionine  $0.26 \times 10^{-3}$  and  $1 \times 10^{-3}$  M, respectively, 2.0 micromoles of creatine were formed per gm. of dry weight of liver per hour; with  $0.78 \times 10^{-3}$  and  $1 \times 10^{-2}$  M guanidoacetic acid and methionine, respectively, the rate was 5.2 micromoles. Intermediate yields of creatine were obtained with intermediate concentrations of methyl acceptor and donor.

ATP added in initial concentrations of 0.003, 0.0015, and 0.00075 M gave the same yields of creatine, and twice that when no ATP was added.

The rate of creatine formation in guinea pig liver homogenate diminishes with time. Thus guanidoacetic acid and methionine gave with ATP alone in 1, 2, and 4 hours 5.9, 8.9, and 8.9 micromoles respectively per gm. (dry weight) of tissue; with ATP plus  $\alpha$ -ketoglutarate the corresponding values were 6.3, 10.4, and 12.1 micromoles; and with adenylic acid plus  $\alpha$ -ketoglutarate 7.3, 9.5, and 10.9 micromoles. In rat liver slices the rate continues nearly unslackened for 6 hours (9). Nearly all of the experiments with guinea pig liver homogenate were run for 1 hour, as more than half the amount formed in 4 hours was obtained in 1 hour and the complications of secondary reactions were lessened.

With rat liver slices, the addition of ATP to the reaction mixture neither accelerated the transmethylation nor did it relieve the inhibiting effect of anaerobiosis. In view of the results obtained with guinea pig liver homogenate the ineffectiveness of ATP with liver slices is to be ascribed to the inability of ATP to penetrate the liver cells.

Table III is a summary of experiments designed to ascertain the relative speeds with which isomers and oxidized derivatives of methionine methylate guanidoacetic acid in guinea pig liver homogenate. L-Methionine had twice the activity of its  $\alpha$ -keto analogue and 5 times that of L-methionine sulfoxide. When the initial concentration of the sulfoxide was reduced 10-fold, to  $1 \times 10^{-3}$  M, it had no activity in the 1 hour period of the test. D-Methionine, L-methionine sulfone, and L-dehydromethionine were either only slightly active or inactive. DL-Methionine was slightly more active than L-methionine. The activity of the  $\alpha$ -keto and sulfoxide derivatives of methionine can be accounted for by inferring their prior conversion to L-methionine; less than 10 per cent conversion would suffice to account for the creatine they formed.

All of the compounds in Table III had been tested as possible methyl donors to guanidoacetic acid with rat liver slices by Handler and Bernheim (10) and by ourselves.<sup>3</sup> D-Methionine had about half the potency of L-methionine, and its effectiveness was nullified by 0.01 M benzoate, which inhibits D-amino acid oxidase (10). The  $\alpha$ -keto analogue of methionine was as effective as L-methionine. Handler and Bernheim found the sulfoxide and sulfone of methionine to be ineffective. We found the sulfoxide and L-dehydromethionine to have 25 to 50 per cent the effectiveness of L-methionine and the sulfone to be ineffective.

TABLE III

*Relative Speeds of Methylation of Guanidoacetic Acid to Creatine by Methionine Isomers and Derivatives*

Volume, 3 ml. 1 ml. of homogenate consisting of 1 part of liver and 2 parts of buffer. Initial concentrations of reactants, guanidoacetic acid  $1 \times 10^{-3}$  M, ATP (purity 93 per cent)  $1.5 \times 10^{-3}$  M,  $\alpha$ -ketoglutarate  $1 \times 10^{-2}$ . The experiment was run for 1 hour at 38° under oxygen.

The figures given are the averages of triplicates expressed as micromoles of creatine.

	Creatine formed
L-Methionine* (0.01 M).....	5.5
D-Methionine† (0.01 " ).....	0.9
DL-Methionine (0.1 M).....	6.3
L-Methionine sulfoxide (0.01 M).....	1.2
"      "      (0.001 " ).....	0
L-Methionine disulfoxide (sulfone) (0.01 M).	-0.1
L-Dehydromethionine (0.01 M).....	0.4
$\alpha$ -Ketomethiol butyrate (0.01 M).....	2.9

\*  $[\alpha]_D^{25} = -7.5^\circ$ .

†  $[\alpha]_D^{25} = +8.76$ .

*In vivo* DL- is as effective as L-methionine for growth and lipotropic purposes, as is also the sulfoxide (24-26). The  $\alpha$ -keto analogue can replace methionine for growth (16). D-Methionine is about as active as the L and D forms lipotropically. Methionine sulfone cannot replace methionine in the diet for growth (27).

All of the observations on the activity of the different derivatives of methionine (including our observation of the positive activity of methionine sulfoxide) in homogenates, liver slices, and *in vivo* are consistent. And they are in accord with the view that L-methionine is the active form of methionine in creatine formation, and that the activity of derivatives is proportional to their prior conversion to L-methionine.

<sup>3</sup> Unpublished observations.

All of the results exclude an oxidation product of methionine as the immediate methyl donor to guanidoacetic acid. They, therefore, also exclude oxidation of methionine as the basis of the dependence of the reaction in liver slices on oxygen, and the participation of ATP in such an oxidation. Another possible explanation of the dependence on oxygen and of the need of ATP was that the immediate methyl acceptor may be N-phosphoguanidoacetic acid, and that the phosphorylation is effected by ATP, analogous to that of creatine. In experiments to test this possibility with rat liver slices and rat and guinea pig liver homogenates it was found that N-phosphoguanidoacetic acid was in no case as effective as guanidoacetic acid

TABLE IV

*Methylation of Guanidoacetic Acid to Creatine in Guinea Pig Liver Homogenate, Effect of Anaerobiosis*

Volume, 3.5 ml. 1 ml. of homogenate consisting of 1 part of liver and 2 parts of buffer. Initial concentrations of reactants, guanidoacetic acid  $1.5 \times 10^{-3}$  M, DL-methionine  $1 \times 10^{-2}$  M, ATP (purity 93 per cent)  $1.5 \times 10^{-3}$  M, adenylic acid  $1.3 \times 10^{-3}$  M,  $\alpha$ -ketoglutarate  $1 \times 10^{-2}$  M, fumarate  $1 \times 10^{-2}$  M. The pH of all the solutions was adjusted to 7.4. The experiment was run for 1 hour at 38° under either oxygen or nitrogen.

The figures given are the averages of triplicates expressed as micromoles of creatine formed per gm. (dry weight) of liver per hour.

Additional metabolite	ATP		Adenylic acid	
	Oxygen	Nitrogen	Oxygen	Nitrogen
	5.9	0.9	0.7	0.2
$\alpha$ -Ketoglutarate.....	7.1	2.2	6.6	1.8
Fumarate.....	6.6	1.2	7.2	0.2
$\alpha$ -Ketoglutarate + fumarate.....	7.3	2.3	7.2	1.8

with or without added ATP, the latter with or without  $\alpha$ -ketoglutarate or fumarate. All of the results indicated that N-phosphoguanidoacetic acid was dephosphorylated before it was methylated to creatine.

If the dependence of the reaction in liver slices on oxygen were solely for the procurement of ATP, it would be expected that the initial addition of ATP to guinea pig liver homogenate would relieve the inhibitory effect of anaerobiosis to some extent. It would not be expected that the inhibition would be entirely relieved, because ATP is hydrolyzed and used in other ways during the incubation, and anaerobic dismutative oxidations will not reconstitute ATP as rapidly as when coupled with the more rapid and more extensive aerobic oxidations.

Table IV is a summary of results of experiments designed to test the foregoing hypothesis. When the reaction mixture contained only ATP in

addition to guanidoacetic acid and methionine, the inhibition of anaerobiosis was 85 per cent. This figure is not significantly different from that obtained with rat liver slices, which in a number of experiments ranged from 90 to 84 per cent.<sup>3</sup>

As was to be expected from previous experiments, addition of adenylic acid alone to the reaction mixture with homogenate gave very little creatine

TABLE V

*Effect of Oxidation Inhibitors on Methylation of Guanidoacetic Acid by Methionine in Rat Liver Slices (without Added ATP or Fumarate) and in Guinea Pig Liver Homogenate (with Added ATP and Fumarate)*

The experiments with rat liver slices were carried out in Krebs-Henseleit Ringer's solution (29) under 95 per cent oxygen and 5 per cent carbon dioxide at 38° for 4 hours. The initial concentrations of guanidoacetic acid and of DL-methionine were  $1.5 \times 10^{-4}$  M and  $4.5 \times 10^{-4}$  M respectively. The volume was 4 ml. The pH of all solutions was adjusted to 7.4. The dry weight of liver tissue in each reaction vessel ranged from 20 to 30 mg. The figures given are the averages of three experiments in which every experimental point and the controls were run in triplicate.

In the experiment with guinea pig liver homogenate the volume was 3 ml., 1 ml. of homogenate consisting of 1 part of liver and 2 parts of buffer (5). Initial concentrations of reactants, guanidoacetic acid  $7.5 \times 10^{-4}$  M, DL-methionine  $1 \times 10^{-2}$  M, fumarate  $1 \times 10^{-2}$  M, ATP (purity 93 per cent)  $1.5 \times 10^{-3}$  M. The pH of all solutions was adjusted to 7.4. These experiments were run for 1 hour at 38° under oxygen. The figures given are the averages of triplicates.

The figures represent the creatine formed, expressed as per cent of yield without inhibitor.

Inhibitor	Rat liver slices	Guinea pig liver homogenate
Arsenate (0.001 M).....	100	100
Arsenite (0.001 " ).....	80	98
Azide (0.001 M).....	11	74
Fluoride (0.02 M).....	95	100
Malonate (0.05 M).....		58
Malonate (0.05 M).....	100	126
Anaerobiosis.....	15	31

formation under oxygen and nearly none under nitrogen. When  $\alpha$ -ketoglutarate and ATP were added to the reaction mixture with homogenate, the inhibitory effect of anaerobiosis was only 69 per cent; and with  $\alpha$ -ketoglutarate and adenylic acid 73 per cent. Fumarate was less effective than  $\alpha$ -ketoglutarate in counteracting anaerobiosis; added with ATP the inhibition was 78 per cent and with adenylic acid 97 per cent.

In general the same results were obtained with oxidation inhibitors as with anaerobiosis (Table V). Included in Table V are the effects of oxidation inhibitors on rat liver slices. The most effective among the inhibitors

tried were anaerobiosis and arsenite. The inhibition of the latter was largely, but not entirely, relieved in the homogenate by the addition of ATP and fumarate. The difference between liver slices and homogenate in the degree of inhibition by arsenite may be taken as evidence that the mechanism of arsenite inhibition in slices is inhibition of ATP formation as a consequence of inhibition of oxidations.

The inhibition by fluoride and acceleration by malonate (Table V) are at present unexplained.

The effect of malonate on the transmethylation appears to be complex, and there is a suggestion in the results with malonate (Table VI) that

TABLE VI

*Effect of Malonate on Methylation of Guanidoacetic Acid to Creatine in Guinea Pig Liver Homogenate*

Volume, 3 ml. 1 ml. of homogenate consisting of 1 part of liver and 2 parts of buffer. Initial concentrations of reactants, guanidoacetic acid  $1.5 \times 10^{-4}$  M, DL-methionine  $1 \times 10^{-2}$  M,  $\alpha$ -ketoglutarate  $1 \times 10^{-2}$  M, fumarate  $1 \times 10^{-2}$  M, ATP (purity 93 per cent)  $1.5 \times 10^{-3}$  M, adenylic acid  $1 \times 10^{-3}$  M, malonate  $5 \times 10^{-2}$  M. The pH of all solutions was adjusted to 7.4. The experiment was run for 1 hour at 38° in oxygen. The figures given are the averages of triplicates, expressed as micromoles of creatine formed per gm. (dry weight) of liver per hour.

	Added metabolite	Without malonate	With malonate
Experiment A	ATP	7.05	3.1
	" + $\alpha$ -ketoglutarate	7.7	4.5
	Adenylic acid + $\alpha$ -ketoglutarate	7.5	3.5
Experiment B	ATP	4.1	2.4
	" + fumarate	5.9	7.5
	Adenylic acid + fumarate	5.1	7.6

oxygen may be required for purposes in addition to the provision of ATP. Guanidoacetic acid and methionine with additions of either ATP alone, ATP plus ketoglutarate, or adenylic acid plus  $\alpha$ -ketoglutarate creatine formation was inhibited by malonate. When fumarate was substituted for  $\alpha$ -ketoglutarate, malonate accelerated the transmethylation.

#### DISCUSSION

The results in Tables IV, V, and VI are open to two interpretations. One is that anaerobiosis, arsenite, fluoride, and malonate inhibit the formation of ATP and thus of creatine through inhibition of oxidations. The other interpretation is that oxygen is necessary to the transmethylation for a number of functions: one is the indirect one of providing ATP; another may be the production of a metabolite reactant necessary for the transmeth-

ylation; still a third may be that oxygen is necessary to support an oxidative step in the over-all transmethylation reaction. The second interpretation as it stands is little more than conjecture. It is prompted by the difference between the effects of  $\alpha$ -ketoglutarate and fumarate, as is shown in Table VI.

We are at present engaged in a purification of the transmethylating enzyme system. When a satisfactory degree of purification is attained, the above alternative interpretations will be examined further. Until then, it seems unprofitable to discuss this aspect of the problem.

Regarding the precise function of ATP, the experimental results obtained exclude participation of ATP in either the oxidation of L-methionine or in the phosphorylation of guanidoacetic acid. Among the possibilities which remain are (1) formation of an intermediate by the condensation of L-methionine and guanidoacetic acid which is phosphorylated by ATP to creatine and a derivative of homocysteine, analogous to the cleavage of cystathionine (28); or (2) L-methionine is phosphorylated by ATP and the phosphorylated L-methionine is the immediate methyl donor to guanidoacetic acid. These possibilities are now under investigation.

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#### SUMMARY

1. The methylation of guanidoacetic acid by methionine proceeds in guinea pig liver homogenate. The rate, on the average, is double that in rat liver slices.

2. ATP is necessary for the transmethylation, and its formation is one of the reasons for the dependence of the transmethylation on oxidation reactions in liver slices and to a lesser extent in guinea pig liver homogenate.

3. The inhibition of the transmethylation by anaerobiosis or arsenite is partially relieved by ATP plus either  $\alpha$ -ketoglutarate or fumarate.

4. Regarding the mechanism of the participation of ATP in the transmethylation the following two possibilities have been excluded: oxidation of L-methionine and phosphorylation of guanidoacetic acid.

5. Evidence is presented that the methyl donor is either L-methionine or a non-oxidative, possibly a phosphorylated, derivative.

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# THE SYNTHESIS OF 5-CHLOROURACIL AND 5-BROMOURACIL NUCLEOSIDES\*

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Kuhn and coworkers (1) found that the substitution of chlorine atoms for the methyl groups of riboflavin produced a specific metabolite antagonist for riboflavin. A similar structural change resulted in an antagonist of thymine. Hitchings and coworkers (2) reported that 5-chlorouracil and 5-bromouracil inhibited the growth of *Lactobacillus casei* and that either thymine or folic acid counteracted this inhibition. Whether 5-chlorouracil and 5-bromouracil nucleosides would be more effective thymine antagonists seemed to present an interesting problem. To solve this, 5-chloro- and 5-bromouracil nucleosides of D-ribose, D-arabinose, D-glucose, and D-galactose were prepared.

The 5-bromouracil nucleosides were prepared from the synthetic nucleosides by the method given by Hilbert and Johnson (3) for the preparation of 1-glucosyl-5-bromouracil. An alternate method in which 1-acetoglycosyl-4-ethoxyuracil, dissolved in carbon tetrachloride, was treated with bromine and then hydrolyzed was less satisfactory and gave poorer yields.

The 5-chlorouracil nucleosides were prepared from either the glycosyluracils or from the intermediate 1-acetoglycosyl-4-ethoxy nucleosides by adding anhydrous chlorine in carbon tetrachloride to the desired nucleoside dissolved in glacial acetic acid. The position of the halogeno substitution was established by hydrolysis of the 1-D-arabinosyl-5-chlorouracil to the known 5-chlorouracil. The position of the chlorine and bromine in the other compounds was assumed by analogy. The preparation from the deacetylated free nucleosides with a 5 to 10 per cent excess of chlorine, as described for the synthesis of 1-D-ribosyl-5-chlorouracil, is undoubtedly the method of choice.

The methods of syntheses of these halogenated nucleosides are reported in this paper. Results of the microbiological studies will be presented elsewhere.

## EXPERIMENTAL

*Acetobromo Sugars*—These compounds were prepared according to the published methods (4-6), as modified by the present authors (7).

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*2,4-Diethoxypyrimidine*—The directions of Hilbert and Johnson (8) were followed for the preparation of 2,4-diethoxypyrimidine from 2,4-dichloropyrimidine.

*1-D-Glucosyluracil*—This compound was prepared by the method of Hilbert and Johnson (3), except that diethoxypyrimidine was substituted for dimethoxypyrimidine.

*1-D-Arabinosyluracil*—1-D-Arabinosyluracil, m.p. 250–252°, was synthesized following the procedure given by Hilbert (9) for the preparation of 1-L-arabinosyluracil. The specific rotation was  $[\alpha]_D^{25} = -88.4^\circ$  ( $c = 2$  in  $H_2O$ ).

*1-D-Galactosyluracil*—The method of Hilbert (9) was followed for the synthesis of this nucleoside.

*1-D-Ribosyluracil*—A modification of the procedure of Hilbert and Rist (10) was developed for the synthesis of 1-D-ribosyluracil. After separation of 2-triacetoribosido-4-ethoxypyrimidine, as described by Hilbert and Rist, the filtrate was cooled in a  $CO_2$ -acetone freezing mixture. The amorphous material, m.p. 60–66°, which separated (8 gm. from 13 gm. of acetobromoribose and 16 gm. of 2,4-diethoxyuracil) was hydrolyzed in HCl-methanol. After removal of the solvents, the residue was crystallized from absolute ethanol. Yield, 27.8 per cent, m.p. 257°.

*1-D-Glucosyl-5-bromouracil*—This compound was prepared according to the procedure of Hilbert and Johnson (3).

*1-D-Ribosyl-5-bromouracil*—Ribosyl-5-bromouracil was prepared from D-ribosyluracil by the procedure of Hilbert and Johnson (3). Yield, 48.5 per cent. The specific rotation was  $[\alpha]_D^{25} = -61.8^\circ$  ( $c = 2$  in  $H_2O$ ).

*1-D-Arabinosyl-5-bromouracil*—A 10 per cent excess of bromine was added to a solution of 1-acetoarabinosyl-1,2-dihydro-2-oxo-4-ethoxypyrimidine dissolved in dry carbon tetrachloride. After standing overnight at room temperature the solvent and excess bromine were removed *in vacuo*. The yellow residue was dissolved in absolute ethanol and the solution was concentrated to dryness by heating in an oil bath. The addition of alcohol and subsequent evaporation to dryness were repeated until the residue was white or slightly yellow. Hydrolysis of this residue in absolute methyl alcohol and HCl yielded an incompletely brominated product. After another treatment with bromine, with the procedure of Hilbert and Johnson (3), a product having the correct analysis for bromine was obtained. Yield, 86 per cent. The product was recrystallized from an alcohol and water solution, m.p. 260°. The optical rotation was  $[\alpha]_D^{25} = -27.7^\circ$  ( $c = 2$  in  $H_2O$ ).

$C_8H_{11}O_6N_2Br$ .	Calculated.	C 33.43, H 3.43, N 8.63, Br 24.25
323.13	Found.	" 33.76, " 3.65, " 8.74, " 24.60

*1-D-Galactosyl-5-bromouracil*—This nucleoside derivative was prepared by the method used for the synthesis of 1-D-arabinosyl-5-bromouracil, and the product (68 per cent) was obtained by dissolving the reaction mixture in an equal weight of hot absolute alcohol followed by the addition of 3 volumes of chloroform. On cooling, the product, 1-D-galactosyl-5-bromouracil, precipitated as a white amorphous solid but was not obtained in a pure state and had an indefinite melting point.

$C_{16}H_{15}O_7N_2Br$ .	Calculated.	N 7.94, Br 22.5
353.15	Found.	" 8.49, " 21.1

*1-D-Glucosyl-5-chlorouracil*—5 gm. of 1-acetoglucosyl-1,2-dihydro-2-oxo-4-ethoxypyrimidine were dissolved in 300 cc. of dry carbon tetrachloride and 25 cc. of glacial acetic acid. A 2 per cent excess (0.767 gm.) of chlorine in 50 cc. of dry carbon tetrachloride was added to this solution at room temperature. The mixture was allowed to stand overnight and the solvents were completely removed under reduced pressure at 40°. The residue was dissolved in 80 cc. of dry methanol and 9.5 cc. of methanol containing 36 per cent by weight of HCl were added. After standing for 3 days the solvents were removed *in vacuo* at 35–40°. The residue was recrystallized from 95 per cent alcohol. Since the chlorine analysis of this product was low it was again treated with a 10 per cent excess of chlorine in carbon tetrachloride, yielding a product which after crystallization had the correct chlorine content. The yield was 2.0 gm. (63 per cent) of white prisms, m.p. 263–264°. The optical rotation was  $[\alpha]_D^{25} = +13.9^\circ$  ( $c = 2$  in  $H_2O$ ).

$C_{16}H_{15}O_7N_2Cl$ .	Calculated.	C 38.90, H 4.24, N 9.06, Cl 11.48
308.69	Found.	" 39.58, " 4.47, " 9.19, " 11.25

*1-D-Arabinosyl-5-chlorouracil*—The procedure already described for the preparation of 1-D-glucosyl-5-chlorouracil was followed for the chlorination of 1-D-acetoarabinosyl-1,2-dihydro-2-oxo-4-ethoxypyrimidine. The product was low in chlorine and was again chlorinated as described for the glucosyl nucleoside. The yield from 4 gm. of the intermediate acetylated nucleoside was 2.5 gm. (89 per cent), m.p. 258°. The optical rotation was  $[\alpha]_D^{25} = -50.4^\circ$  ( $c = 2$  in  $H_2O$ ).

$C_{16}H_{15}O_7N_2Cl$ .	Calculated.	C 38.79, H 3.95, N 10.05, Cl 12.72
278.67	Found.	" 39.09, " 4.42, " 10.10, " 12.80

*1-D-Galactosyl-5-chlorouracil*—This compound was synthesized by the procedure described for the preparation of glucosyl-5-chlorouracil. The product was isolated in the same manner as galactosylthymine (7). Analy-

sis showed that the product (53 per cent), which had an indefinite melting point, was nearly pure.

$C_{10}H_{13}O_7N_2Cl$ .	Calculated.	C 38.90, H 4.24, N 9.06
308.69	Found.	" 38.60, " 4.24, " 9.50

*1-D-Ribosyl-5-chlorouracil*—This compound was synthesized by the addition of a 6.5 per cent excess of chlorine in carbon tetrachloride to a glacial acetic acid solution of 1-D-ribosyluracil. After standing overnight the solvents were removed under reduced pressure at 35°, and the residue

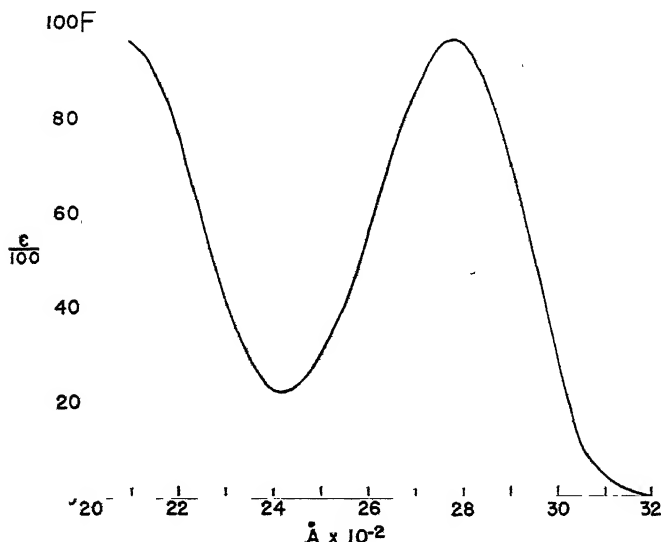


FIG. 1. Ultraviolet absorption spectrum of 1-D-ribosyl-5-bromouracil. The wavelength is plotted against the molecular extinction ( $\epsilon$  ( $\epsilon = E \times \text{mol. wt.}$ )/( $cd$ ), where  $E$  = extinction, mol. wt. = molecular weight of compound,  $c$  = concentration in gm. per liter, and  $d$  = cell thickness in cm.)

recrystallized from absolute alcohol, m.p. 245°; 0.4 gm. of 1-D-ribosyl-5-chlorouracil (40 per cent) was obtained from 0.7 gm. of D-ribosyluracil. The optical rotation was  $[\alpha]_D^{25} = -87.3^\circ$  ( $c = 2$  in  $H_2O$ ).

$C_8H_{11}O_6N_2Cl$ .	Calculated.	C 38.79, H 3.95, N 10.05, Cl 12.72
278.67	Found.	" 39.68, " 4.08, " 10.15, " 12.76

*5-Chlorouracil from Arabinosyl-5-chlorouracil*—0.5 gm. of arabinosyl-5-chlorouracil was refluxed with 25 cc. of concentrated HCl for 12 hours. The mixture was concentrated to dryness and the 5-chlorouracil crystallized from water, m.p. 304°; mixed m.p. with 5-chlorouracil showed no depression.

*Ultraviolet Absorption Spectra of Uracil Nucleosides*—The ultraviolet absorption spectra of the uracil nucleosides of D-ribose, D-arabinose, and D-glucose and the corresponding 5-chloro and 5-bromo derivatives were determined with a Beckman spectrophotometer with a hydrogen discharge tube as the source of light. The maxima and minima are listed in Table I. The absorption obtained is illustrated by the curve of 5-bromoribosyluracil of Fig. 1.

The authors are indebted to Mr. Jack Fox for technical assistance and to Mr. Frank Rainwater for some of the analytical results.

TABLE I  
*Maximum and Minimum Ultraviolet Absorption of Uracil Nucleosides and Their Bromo and Chloro Derivatives*

	Maximum	Minimum
	$\lambda$	$\lambda$
1-D-Arabinosyluracil .....	2580	2280
1-D-Glucosyluracil .....	2580	2280
1-D-Ribosyluracil .....	2620	2300
Arabinosyl-5-chlorouracil .....	2740	2380
Glucosyl-5-chlorouracil .....	2720	2360
Ribosyl-5-chlorouracil .....	2770	2360
Arabinosyl-5-bromouracil .....	2760	2410
Glucosyl-5-bromouracil .....	2760	2410
Ribosyl-5-bromouracil .....	2790	2420

#### SUMMARY

5-Chlorouracil and 5-bromouracil nucleosides of D-ribose, D-arabinose, D-glucose, and D-galactose were synthesized by direct chlorination or bromination of the corresponding synthetic nucleosides.

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A NEW SYNTHESIS OF  $\beta$ -2-FURYLALANINE. SOME  
DERIVATIVES OF  $\beta$ -2-FURYLALANINE  
AND  $\beta$ -2-THIENYLALANINE\*

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Recent work (1-3) has shown that  $\beta$ -2-thienylalanine inhibits microbial growth by virtue of its antagonism to phenylalanine. This antagonistic activity resulted from the replacement of the vinylene group by the isosteric sulfur atom.

These results created an interest in studying the effect of amino acids containing simple heterocyclic nuclei other than thiophene on the growth of microorganisms. One of the amino acids selected for such a study was  $\beta$ -2-furylalanine. Although this compound has been known for nearly 40 years, it has not been investigated microbiologically. It was first prepared by Flatow (4) by the hippuric acid method (5). Sasaki (6) obtained it in good yield via the furfural-diketopiperazine condensation product. At approximately the same time Gränacher (7) extended the rhodanine method for the preparation of amino acids to furylalanine, and, more recently, Deulofeu (8) used hydantoin as a convenient reagent for its synthesis.

Although these methods give fairly good yields, it was decided to study the preparation of furylalanine by a variation of the acetamidomalonic ester method (9, 10) which had been found very convenient in the synthesis of thienylalanine (11). Furfuryl chloride was condensed in the usual manner with ethyl acetamidomalonate in 60 to 70 per cent yield, and in 55 to 65 per cent yield with ethyl cyanoacetamidoacetate, a method developed more recently (12). The latter product was not obtained in crystalline form, but remained an oil after many weeks of standing.

It was found that ethyl 2-furylmethyl acetamidomalonate, when subjected to basic hydrolysis, required stepwise degradation to the amino acid, as already observed for the indole analogue (9, 10). In addition only a small yield of furylalanine, contaminated by inorganic matter, was realized. Acid hydrolysis was not employed because of the sensitivity of the furan nucleus. For these reasons, ethyl  $\alpha$ -acetamido- $\alpha$ -cyano- $\beta$ -(2-furyl)-propionate, though an unpurified oil, was employed as an intermediate. Basic hydrolysis of this substance gave a 40 to 50 per cent yield of furylalanine.

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For purposes of identification, the phenylurea and the new N-carbobenzoy derivatives of furylalanine were prepared. Since there are no reported derivatives by which thienylalanine can be identified, the N-carbobenzoy and phenylurea derivatives of thienylalanine were also prepared.

The microbiological work on  $\beta$ -2-furylalanine will be reported in a separate paper.

#### EXPERIMENTAL<sup>1</sup>

*Ethyl 2-Furylmethyl Acetamidomalonate*—To a solution of sodium ethoxide, prepared by the addition of 2.3 gm. of sodium to 100 ml. of absolute ethanol, were added 21.8 gm. of ethyl acetamidomalonate followed by 12 gm. of freshly distilled furfuryl chloride (13). Heat was developed immediately and a precipitate of sodium chloride began to form. The mixture was refluxed for 4 hours and evaporated to a small volume *in vacuo*. The residual syrup was diluted with 100 ml. of cold water and chilled overnight. When the product was broken up with a stirring rod and filtered, 21 gm. of orange-colored material were obtained. It was redissolved in 40 ml. of ethanol, decolorized with charcoal, chilled, and diluted with 100 ml. of water. 16 gm. of white needles, m.p. 84°, separated; the mother liquors yielded an additional 3 gm. of less pure material. It was soluble in ethanol, acetone, benzene, and ether, partially soluble in hot water, and insoluble in petroleum ether. For analysis, the product was recrystallized once more from an ethanol-water mixture without further change in melting point.

$C_{14}H_{20}NO_6$  (297.3). Calculated, N 4.71; found, N 4.78

*$\beta$ -2-Furylalanine*—4.6 gm. of clean sodium were dissolved in 200 ml. of absolute ethanol. To the solution were added first 34 gm. of ethyl cyanoacetamidoacetate and then, in one portion, 24 gm. of freshly distilled furfuryl chloride. After the initial reaction had subsided, the mixture was refluxed for 2 hours, cooled, evaporated to a syrup *in vacuo*, diluted with 300 ml. of water, and chilled overnight. The water was decanted; the residue was dissolved in 60 ml. of ethanol, treated with charcoal, filtered, cooled, diluted with water, and chilled. 25 gm. of viscous material were obtained which did not crystallize after standing for several weeks.

The oil was refluxed with 100 ml. of 25 per cent sodium hydroxide for 19 hours. The hydrolysis mixture was treated with charcoal, filtered, neutralized with concentrated hydrochloric acid, and filtered while hot. The material remaining on the filter paper was washed with hot water. The combined filtrate and washings were acidified to pH 5 and chilled overnight. By next morning 8 gm. (24 per cent based on furfuryl chloride)

<sup>1</sup> All melting point values reported in this paper are uncorrected.

of slightly colored furylalanine had settled out. The product was filtered, washed with a little ethanol, and melted at 260° after recrystallization from 50 per cent ethanol. A small amount of less pure material was recovered from the mother liquors by evaporating to dryness *in vacuo*, extracting the residue with 50 per cent ethanol, decolorizing with charcoal, and chilling.

$C_7H_9NO_3$  (155.2). Calculated, N 9.03; found, N 8.49

For identification, the phenylurea was prepared according to the directions given by Meyer (14). The white crystals melted at 163–164° (capillary) and at 178° within 3 seconds on a Dennis melting point bar. This compares with 162–163° given by Sasaki (6).

*N*-Carbobenzoxymethyl- $\beta$ -2-furylalanine—465 mg. of furylalanine were treated with carbobenzoxymethyl chloride in the usual manner (15). The ether-extracted aqueous solution was carefully acidified with concentrated hydrochloric acid in the cold and chilled overnight. The derivative was purified by redissolving in base, decolorizing, acidifying in the cold, allowing to stand for several hours, filtering, and washing with water. The yield was 480 mg. (55 per cent), m.p. 89°; the neutral equivalent found was 288.

$C_{15}H_{15}NO_5$  (289.3). Calculated, N 4.84; found, N 4.66

*N*-Carbobenzoxymethyl- $\beta$ -2-thienylalanine—This derivative was prepared in essentially the same manner as the furan analogue from 510 mg. of thienylalanine (11). The yield of white crystals, m.p. 101°, was 490 mg. (54 per cent); the neutral equivalent was found to be 303.

$C_{15}H_{13}NO_4S$  (305.3). Calculated, N 4.59; found, N 4.64

*Phenylurea Derivative of  $\beta$ -2-Thienylalanine*—0.85 gm. of thienylalanine dissolved in the calculated amount of sodium hydroxide was treated with 0.65 gm. of phenyl isocyanate in the usual manner (14). The crude product obtained in quantitative yield was recrystallized twice from 50 per cent ethanol and melted at 165–166° (capillary) and at 182° within 3 seconds on the melting point bar. The neutral equivalent was found to be 294.

$C_{14}H_{11}N_2O_3S$  (290.3). Calculated, N 9.58; found, N 9.53

#### SUMMARY

$\beta$ -2-Furylalanine has been prepared conveniently in 24 per cent yield by the reaction of furfuryl chloride with ethyl cyanoacetamidoacetate followed by basic hydrolysis.

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# INHIBITION OF THE GROWTH OF STAPHYLOCOCCUS AUREUS BY SOME DERIVATIVES OF GLUTAMIC ACID

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As has previously been reported (1), L-N-( $\gamma$ -glutamyl)-methylamine and L-N-( $\gamma$ -glutamyl)-ethylamine have been obtained by treating L-pyrrolidonecarboxylic acid with aqueous solutions of methylamine and ethylamine respectively. In a similar manner, we succeeded in preparing the following  $\gamma$ -alkyl derivatives of glutamic acid from L- or DL-pyrrolidonecarboxylic acid and from the corresponding amines: DL-N-( $\gamma$ -glutamyl)-ethylamine, L-N-( $\gamma$ -glutamyl)-*n*-butylamine, L-N-( $\gamma$ -glutamyl)-ethanolamine, and DL-N-( $\gamma$ -glutamyl)-ethanolamine. All of these compounds have abnormally high Van Slyke amino nitrogen values, such as those characteristic of glutamine and of  $\gamma$ -methylamide and  $\gamma$ -ethylamide of L-glutamic acid (1).

Substances structurally related to metabolites may interfere with their normal function in living cells (2-5). It occurred to us, therefore, the  $\gamma$ -alkylamides of glutamic acid might act as antagonists of either glutamic acid or glutamine in the process of bacterial growth. *Staphylococcus aureus* was chosen as the test organism for a study of this question. We have been able to demonstrate that either DL-N-( $\gamma$ -glutamyl)-ethylamine, L-N-( $\gamma$ -glutamyl)-ethanolamine, or DL-N-( $\gamma$ -glutamyl)-ethanolamine strongly inhibits the growth of this microorganism, and that this inhibition is counteracted by L-glutamic acid.

## EXPERIMENTAL

*Preparation of Glutamic Acid Derivatives*—L-Pyrrolidonecarboxylic acid was prepared as previously described (1).

DL-Pyrrolidonecarboxylic acid was obtained by heating L-glutamic acid in an oil bath at a bath temperature of 190–200° for 2½ hours. The pyrrolidonecarboxylic acid thus formed was recrystallized from hot water.

DL-N-( $\gamma$ -Glutamyl)-ethylamine was obtained in the same way as the L derivative (1). Yield, 0.8 to 0.9 gm. from 6 gm. of DL-pyrrolidonecarboxylic acid. For analysis, the substance was dissolved in a small quantity of water and precipitated by the addition of alcohol. It was then dried *in vacuo* over phosphorus pentoxide in a drying apparatus heated with boiling chloroform; m.p. 200° (uncorrected).

$C_7H_{14}O_3N_2$ . Calculated. C 48.2, H 8.1, N 16.1  
 174.2 Found. " 47.8, " 7.9, " 16.2, amino N 14.8 (5  
 min. shaking in a Van Slyke volumetric apparatus)

*L-N-( $\gamma$ -Glutamyl)-*n*-butylamine*<sup>1</sup>—4 gm. of *L*-pyrrolidonecarboxylic acid were treated with 36 gm. of 33 per cent aqueous *n*-butylamine solution for 20 to 25 days at 37° in a sealed glass tube. At the end of this time the fluid was filtered, transferred to a crystallizing dish, and placed in a vacuum desiccator over sulfuric acid at room temperature, as in the case of the methyl and ethyl derivatives (1). The resulting syrup was rubbed with 100 cc. of absolute alcohol, and the mixture was stored in a corked bottle in the refrigerator overnight. The precipitate obtained, which was in the form of colorless, glittering platelets, was filtered by suction, washed with cold absolute alcohol, and dried in a vacuum desiccator over sulfuric acid. Yield, 50 mg.; m.p. 209° (uncorrected).

$C_9H_{18}O_3N_2$ . Calculated. C 53.4, H 9.0, N 13.9  
 202.2 Found. " 53.3, " 8.8, " 14.1, amino N 12.7  
 (Van Slyke apparatus, 5 min. shaking)

*L-N-( $\gamma$ -Glutamyl)-ethanolamine*<sup>2</sup>—7.5 gm. of *L*-pyrrolidonecarboxylic acid were treated with 45 gm. of 33 per cent aqueous ethanolamine solution for 20 to 25 days at 37° in a sealed glass tube. The filtered solution was evaporated to a syrup in a vacuum desiccator over sulfuric acid, either at room temperature or, preferably, at 37°. The syrup was rubbed with 150 cc. of absolute alcohol and stored for several days in a corked flask in the refrigerator. The crystalline, colorless precipitate was filtered by suction, washed with cold absolute alcohol, and dried in a vacuum desiccator over sulfuric acid. Yield, 0.7 gm.; m.p. 176° (uncorrected). For analysis, the substance was dissolved in a little water and was precipitated with alcohol. It was dried *in vacuo* over sulfuric acid and then over phosphorus pentoxide in a drying apparatus heated with boiling chloroform.

$C_7H_{14}O_4N_2$ . Calculated. C 44.2, H 7.4, N 14.7  
 190.2 Found. " 43.8, " 7.5, " 14.6, amino N 12.9  
 (Van Slyke apparatus, 5 min. shaking)

$[\alpha]_D^{15} = +5.4^\circ$  (0.5 per cent aqueous solution).

*DL-N-( $\gamma$ -Glutamyl)-ethanolamine*—This substance was prepared in the same manner as the *L* isomer. Yield, 1.5 gm. from 7.5 gm. of *DL*-pyrrolidonecarboxylic acid; m.p. 175–176° uncorrected.

$C_7H_{14}O_4N_2$ . Calculated. C 44.2, H 7.4, N 14.7  
 190.2 Found. " 44.2, " 7.6, " 14.6, amino N 12.6  
 (Van Slyke apparatus, 5 min. shaking)

<sup>1</sup> With the assistance of Lea Freidinov.

<sup>2</sup> With the assistance of M. Salomonowicz.

### Assay Procedure

**Basal Medium**—The tests were performed in a basal medium of the following composition: glucose 3 gm., casein hydrolysate (vitamin-free) 4 gm., NaCl 2 gm.,  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$  5 gm.,  $\text{KH}_2\text{PO}_4$  0.35 gm.,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.30 gm., tryptophan 10 mg., thiamine 1 mg., nicotinamide 1 mg., biotin 0.01 mg. The solution was brought to 500 cc. volume.

This double strength medium was distributed in 2.5 cc. aliquots in tubes of ordinary size (15 × 165 mm.), to which water or substances to be tested were then added, and the volume was made up to 5 cc. The basal medium was thus reduced to half its original concentration. The medium may be sterilized in two ways, either by adjusting its pH to 7 (to avoid precipitation of magnesium phosphate by heating) and then autoclaving at 15 pounds pressure for 20 minutes, or by autoclaving a medium devoid of glucose and magnesium sulfate at pH 7.4 and subsequently adding these substances aseptically from solutions sterilized separately. Since better growth was obtained at an alkaline pH, the second method of preparation was preferred.

The sodium salts of L-glutamic acid and L- and DL-pyrrolidonecarboxylic acid and the hydrochlorides of ethylamine and ethanolamine were sterilized by autoclaving. (Analyses showed that the substances remained unaffected by this treatment.) The  $\gamma$ -alkylamides of glutamic acid as well as glutamine were sterilized by filtration and added aseptically to the sterile basal medium.

**Assay**—The assays were performed as follows: The tubes were inoculated with 0.1 cc. of a suspension of *Staphylococcus aureus* grown on agar, prepared by diluting a just barely turbid suspension 1000-fold. Such a standard inoculum contains approximately  $10^4$  bacteria. The growth was measured by estimating the turbidity with the aid of an Evelyn type of electrophotometer. With an inoculum diluted to this extent, growth reached its peak only after 35 to 40 hours and was therefore measured after 48 hours of incubation. More prolonged incubation did not alter the results. As is seen from Table I, both DL-N-( $\gamma$ -glutamyl)-ethylamine and L-N-( $\gamma$ -glutamyl)-ethanolamine strongly inhibited the growth of the test organism. 6 mg. of the former and 8 mg. of the latter inhibited growth by about 15 per cent, while 16 and 20 mg., respectively, inhibited growth by 70 per cent or even more. On the other hand, neither the corresponding amines nor pyrrolidonecarboxylic acid caused any noteworthy inhibition. (The amines and pyrrolidonecarboxylic acid were included in the experiments because small quantities of the corresponding amino salts of pyrrolidonecarboxylic acid may be present in the preparations of the alkylamides of glutamic acid.) Similar experiments were carried out with DL-N-( $\gamma$ -

TABLE I

*Effect of Glutamic Acid Derivatives on Growth of Staphylococcus aureus*

Compound added	Concentration per 5 cc.	Photometer reading after 48 hrs. growth
	mg.	
None (sterile medium) . . . . .		100
" (inoculated) . . . . .		43
Ethylamine hydrochloride . . . . .	30	41
Ethanolamine hydrochloride . . . . .	30	39
Sodium salt of L-pyrrolidonecarboxylic acid . . . . .	30	44
" " " DL-pyrrolidonecarboxylic acid . . . . .	30	48
DL-N-( $\gamma$ -Glutamyl)-ethylamine . . . . .	20	83
" . . . . .	16	85
" . . . . .	10	61
" . . . . .	6	51
L-N-( $\gamma$ -Glutamyl)-ethanolamine . . . . .	20	80
" . . . . .	16	77
" . . . . .	12	58
" . . . . .	8	50

TABLE II

*Effect of L-Glutamic Acid and of L-Glutamine on Inhibition of Growth of Staphylococcus aureus by Glutamic Acid Derivatives*

Inhibitor	Concentration per 5 cc.	Glutamic acid (Na salt) or glutamine	Concentration per 5 cc.	Photometer reading after 48 hrs. growth
	mg.		mg.	
None . . . . .		Glutamic acid	0	35
" . . . . .		" "	3	36
" . . . . .		" "	5	36
L-N-( $\gamma$ -Glutamyl)-ethanolamine . . . . .	20	" "	0	82
" . . . . .	20	" "	0.5	65
" . . . . .	20	" "	1	48
" . . . . .	20	" "	2	38
None . . . . .		Glutamine	0.5	36
" . . . . .		" "	1	38
L-N-( $\gamma$ -Glutamyl)-ethanolamine . . . . .	20	" "	0	82
" . . . . .	20	" "	0.5	82
" . . . . .	20	" "	1	83
DL-N-( $\gamma$ -Glutamyl)-ethylamine . . . . .	20	Glutamic acid	0	80
" . . . . .	20	" "	1	79
" . . . . .	20	" "	3	68
" . . . . .	20	" "	5	43

glutamyl)-ethanolamine. The inhibition obtained with several preparations was sometimes equal to and sometimes less marked than that obtained with the L isomer. These differences in activity might possibly be

explained by the assumption that traces of glutamic acid were present in various preparations of both isomers (see below).

Further experiments showed that it was impossible to obtain complete inhibition even with amounts twice as large as those described. Maximum

TABLE III

*Inhibiting Action of Glutamic Acid Derivatives on Growth of Staphylococcus aureus after Varying Periods of Incubation*

Compound added	Concentration per 5 cc.	Photometer reading after				
		21 hrs.	24 hrs.	27 hrs.	32 hrs.	48 hrs.
	mg.					
None (sterile) . . . . .						100
" (inoculated) . . . . .		83	67	53	51	43
L-N-( $\gamma$ -Glutamyl)-ethanolamine . . .	20	84	83	81	82	79
" . . . . .	16	88	81	81	83	78
" . . . . .	12	84	77	70	59	60
D,L-N-( $\gamma$ -Glutamyl)-ethylamine . . .	16	85	86	86	85	85
" . . . . .	10	86	86	87	87	87

TABLE IV

*Effect of Size of Inoculum on Inhibition of Staphylococcus aureus by Glutamic Acid Derivatives*

Compound added	Concentration per 5 cc.	Inoculum	Photometer reading after				
			17 hrs.	20 hrs.	23 hrs.	43 hrs.	72 hrs.
	mg.						
None . . . . .		Standard	82	67	50	46	46
" . . . . .		10-fold	71	52	47	46	46
" . . . . .		100-fold	54	46	46	46	46
D,L-N-( $\gamma$ -Glutamyl)-ethylamine . . .	14	Standard	82	80	83	83	83
" . . . . .	14	10-fold	85	83	86	84	84
" . . . . .	14	100-fold	78	79	81	79	80
L-N-( $\gamma$ -Glutamyl)-ethanolamine . . .	14	Standard	85	81		82	81
" . . . . .	14	10-fold	79	80		82	81
" . . . . .	14	100-fold	80	82		84	84

inhibitions obtained with the medium used varied between 70 and 85 per cent.

*Reversal of Inhibition by Addition of Glutamic Acid*—Table II shows that the inhibition induced by the N-( $\gamma$ -glutamyl)-amines could be overcome by L-glutamic acid, whereas glutamine in the amounts tested proved to be ineffective. Smaller quantities of glutamic acid were required to attain complete reversal in the case of L-N-( $\gamma$ -glutamyl)-ethanolamine than in the case of D,L-N-( $\gamma$ -glutamyl)-ethylamine.



*Inhibiting Effect of Glutamic Acid Derivatives after Varying Periods of Incubation*—Table III shows that neither DL-N-( $\gamma$ -glutamyl)-ethylamine nor L-N-( $\gamma$ -glutamyl)-ethanolamine influenced early growth. After 21 hours, growth in both series, with or without inhibitors, was practically equal. Later, however, growth in the control tubes proceeded rapidly, whereas no change in turbidity was observed in the tubes containing the alkylamides.

*Effect of Size of Inoculum on Inhibition*—The appearance of the peak may be accelerated by the addition of a larger inoculum, but the final degree of inhibition was not affected by even a 100-fold increase of the inoculum (Table IV).

#### DISCUSSION

The fact that the inhibitory action of the  $\gamma$ -glutamylamines on *Staphylococcus aureus* does not become pronounced until after 24 to 27 hours of incubation (Table III) may be explained if it is assumed that the concentration of glutamic acid originally present in the medium is sufficient to overcome the action of the inhibitor, and thereby to permit normal growth. Later, however, as growth proceeds, the glutamic acid is gradually consumed and the action of the antagonists becomes more and more pronounced.

According to Woolley (3), one of the methods of converting an acidic metabolite into an inhibitory agent is by the exchange of some other group for a carboxyl. This type of structural change involves either (Class A) the replacement of the entire carboxyl group by some other group (e.g. nicotinic acid  $\rightarrow$  3-pyridinesulfonic acid) or (Class B) the replacement of  $-\text{COOH}$  by  $-\text{COR}$  (e.g. nicotinic acid  $\rightarrow$  3-acetylpyridine). It has been found by Borek, Miller, Sheiness, and Waelsch (6) and by Waelsch, Owades, Miller, and Borek (7) that sulfoxides derived from methionine and from benzylhomocysteine act as effective antimetabolites of glutamic acid in the growth of *Lactobacillus arabinosus*. Whereas the inhibiting effect obtained by these authors belongs in Class A of the structural changes mentioned above, inhibition of the growth of *Staphylococcus aureus* by  $\gamma$ -alkylamides of glutamic acid obviously falls within Class B.

#### SUMMARY

1. The preparation of the following new derivatives of glutamic acid is described: DL-N-( $\gamma$ -glutamyl)-ethylamine, L-N-( $\gamma$ -glutamyl)-*n*-butylamine, L-N-( $\gamma$ -glutamyl)-ethanolamine, DL-N-( $\gamma$ -glutamyl)-ethanolamine.
2. DL-N-( $\gamma$ -glutamyl)-ethylamine and L- and DL-N-( $\gamma$ -glutamyl)-ethanolamine markedly inhibited the growth of *Staphylococcus aureus*. This inhibition was reversed by L-glutamic acid.

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LIVER REGENERATION AND CYTOCHROME *c* METABOLISM.  
INFLUENCE OF AMOUNT OF TISSUE EXCISED AND OF  
DIET, WITH A NOTE ON ACCOMPANYING  
CHANGES IN LIVER NUCLEIC ACIDS\*

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In a previous communication from this laboratory (2), partial hepatectomy or liver lobectomy in the rat was introduced as a quantitative metabolic technique in the study of cytochrome *c*. The removal of liver substance may be regarded as a "depletion stimulus" to the ensuing proliferative process, affording an opportunity for the quantitative study not only of the appearance or production of substances of metabolic interest in the regenerating tissue, but also of the restorative process itself. The rate of appearance of new cytochrome *c* in the regenerating liver was thus determined during a restoration (postoperative) period of 14 days (2). For historical information, details of the liver lobectomy and the high degree of reproducibility of ratios of lobe weight to total liver mass, reference may be made to the earlier paper (2). In a continuation of this work, the influence of a number of factors upon cytochrome *c* metabolism and liver restoration has been studied. Among the factors investigated and reported here are the effect of the amount of liver excised, the rôle of dietary protein (no protein and high protein diet), and the influence of length of duration of the restoration period. In the following paper results will be presented upon the effect of oxygen deprivation (anoxia, induced by simulated high altitude), and on the influence of injection of cytochrome *c* prior to liver lobectomy and during active tissue restoration. The experimental evidence suggests that cytochrome *c* is an important component in the regenerative process. The nitrogen metabolism of partially hepatectomized rats and the effect of various factors in liver regeneration were independently investigated by Vars, Gurd, and Ravdin (3).

\* This work was carried out under contract between the Office of Naval Research and the University of Pennsylvania. A report was made at the meetings of the Federation of American Societies for Experimental Biology at Chicago, May 18-22, 1947, and an abstract has appeared (1).

### Methods

*Regimen*—Adult albino rats from our laboratory colony, originally of Wistar Institute stock (2), of known age (150 to 200 days) and 180 to 300 gm. in weight were used. Records were kept of the initial weight before the start of the special dietary regimens, of preoperative weight after 13 days on the experimental diet followed by a 24 hour fasting period, and of final weight 4, 6, and 14 days postoperatively, the animals being maintained on the experimental diet for 3, 5, and 13 days respectively, followed by a 24 hour fasting period in each case before sacrifice in the manner previously described (2). It may be emphasized that in the above procedure the "internal control" liver tissue excised at the lobectomy and the final regenerated liver obtained at the termination of the experiment are from animals fasted 24 hours and therefore low in glycogen (of the order of 0.5 to 0.6 per cent). This fasting period provides an additional burden for the animals on the protein-free diet, but is advantageous for accurate cytochrome *c* analyses (2, 4, 5). It appears probable that 13 days on no protein plus 1 day of fasting preoperatively may represent the maximal deprivation stimulus for liver regeneration to which the rat organism may be exposed with any degree of safety.<sup>1</sup>

The diets employed, high and no protein respectively, were synthetic. Each provided the same number of calories, the same salt mixture, and the same full supplement of vitamins. The composition of the *high* (31 per cent) *protein diet* was as follows: sucrose 53.5, Cellu flour 2.0, crude casein (80 to 83 per cent of protein content) 37.5, cod liver oil 3.0, and salt mixture 4.0 parts per hundred. In the *no protein diet* the casein was omitted and the sucrose content was 91 parts per hundred. The salt mixture was slightly modified from that of No. 12 of Jones (6) by doubling the allowance of iron to assure an optimal provision of this element. This salt mixture was chosen since it provides, besides the common inorganic elements, also those required in trace quantities, as Mn, Zn, Cu, and Co, some of which may be involved in hemin metabolism. Per kilo of each dietary mixture the following vitamin supplements were added: thiamine 0.01, riboflavin 0.01, nicotinic acid 0.10, pyridoxine 0.01, calcium pantothenate 0.06, inositol 0.60, *p*-aminobenzoic acid 0.20, and choline chloride 2.0 gm. On an average intake of 6 to 10 gm. of the mixed diets per rat

<sup>1</sup> Whereas on an adequate diet (2) and on the present high protein diet the surgical mortality was zero, on the no protein regimen six of seventeen animals did not survive. Two of these rats died preoperatively during the administration of ether anesthesia; the other four animals in the first few postoperative days. The eleven rats which survived for the whole experimental period of 14 days following liver excision resumed alimentation promptly after the operation. Hence, although the diet is lacking in protein, there is no doubt some virtue in the intake of calories.

per day, approximately 1/100 to 1/150 of the above amounts of each vitamin and approximately 3 to 4 mg. of Fe and 0.04 to 0.05 mg. of Cu were consumed daily.

*Liver Lobectomy*—Except for a slight modification, the excision of the cartilagenous portion of the xiphoid process,<sup>2</sup> the technique for liver lobectomy was as given previously (2). In the present experiments both the left lateral and median lobes (*cf.* (2)) were excised. This represents a removal of 68.4 per cent of the liver, the closely reproducible ratio of (gm. of left lateral + gm. of median lobe)/(gm. of original total liver) = 0.684 having been established for the rats of our colony by Crandall and Drabkin (2). Two quantities, "gm. of liver excised" and "gm. of final total liver," are measured directly (*cf.* (2)). Other values are calculated as follows:

$$\text{Gm. original total liver} = \text{gm. liver excised}/0.684 \quad (1)$$

$$\text{Gm. remaining liver} = (\text{gm. original total liver}) - (\text{gm. liver excised}) \quad (2)$$

$$\text{Gm. new or restored liver} = (\text{gm. final total liver}) - (\text{gm. remaining liver}) \quad (3)$$

$$\text{Fraction restoration or regeneration} = \frac{\text{gm. new or restored liver}}{\text{gm. liver excised}} \quad (4)$$

$$\% \text{ restoration or regeneration} = \text{fraction restoration or regeneration} \times 100 \quad (5)$$

The above relationships may be combined to show the over-all dependency upon the two measured quantities,

Fraction restoration or regeneration =

$$\frac{(\text{Gm. final total liver}) - ((\text{gm. liver excised}/0.684) - (\text{gm. liver excised}))}{\text{Gm. liver excised}} \quad (6)$$

This is our usual method of calculating the amount of tissue regeneration or the amount of new cytochrome *c* which has appeared in the regenerated liver from analyses upon the tissue excised at operation and upon the liver removed at the termination of the experiment. The animals on the no protein diet, in contrast with those on the high protein diet, lost weight during the course of the experiment (Tables I and III). In 2 weeks pre-operatively on the no protein diet the loss of weight averaged approximately 54 gm. (from the data in Table III, with the initial weight before starting the diet corrected for 1 day of fasting). This loss of body substance raises the question of the validity of comparing the results upon the two diets on the common basis of Equation 6. Theoretically a more valid comparison might be obtained by referring regeneration to the mass

<sup>2</sup> This modification affords a freer access to the abdominal cavity and was introduced by Dr. Fraser Gurd of the Harrison Department of Surgical Research. I am highly indebted to Dr. Gurd for most of the surgery and to the above department for the use of their facilities.

of the original liver, prior to initiation of the special diets. In this alternative method of calculation reliance must be placed upon a relationship of liver mass to body mass, which for our 24 hour-fasted rats was established to be gm. of liver =  $0.0262 \times$  gm. of body mass (2). By means of the above factor the following relationship is obtained.

Fraction restoration or regeneration =

$$\frac{(\text{Gm. final total liver}) - ((\text{gm. initial body mass} \times 0.0262) - (\text{gm. liver excised}))}{\text{Gm. liver excised}} \quad (7)$$

Although in our earlier experiments (2) *gm. of original total liver* appeared to be about as reliably obtainable from *gm. of liver excised*/0.684 as from *gm. of initial body mass*  $\times$  0.0262, in the present work, in agreement with the findings of others (7), the relationship of mass of lobes to mass of total liver was the more consistent. I therefore favor calculations by means of Equation 6. While the values obtained by the alternative methods of calculation do not agree closely in some animals, the magnitude of the differences is such that identical conclusions may be reached by either method (2).

*Analytical Procedures*—The concentration of cytochrome *c* in the excised liver and, at the termination of the experiment, in liver, kidney cortex, heart, and skeletal muscle<sup>3</sup> was determined by the direct micro spectrophotometric method of Rosenthal and Drabkin (4). Upon aliquots of the tissues the wet weight to dry weight ratios were obtained as previously described (2). In place of the protein-bound phosphorus index of "cellularity" (5), in the present experiments the concentrations of ribose nucleic acid, PNA, and of desoxyribose nucleic acid, DNA, were determined in appropriate tissue aliquots, according to Schneider (8). These analyses were made with the Klett-Summerson (9) photoelectric photometer, the orcinol reaction and the red ( $\lambda$  660 m $\mu$ ) filter being used for PNA and for DNA the diphenylamine reaction with the green ( $\lambda$  540 m $\mu$ ) filter.<sup>4</sup>

### Results

The data presented in Table I establish the fact that both liver restoration and the amount of appearance of new cytochrome *c* in the regenerating liver are functions of the amount of tissue excised. Appreciably more

<sup>3</sup> As in the previous work (2), no significant changes were found in tissues other than liver after partial hepatectomy. Therefore, data on kidney cortex, heart, and skeletal muscle will not be reported.

<sup>4</sup> The photometric analyses were against standard curves obtained with samples of purified yeast nucleic acid (PNA) and the sodium salt of thymus nucleic acid (DNA), for which I am indebted to Dr. Seymour S. Cohen, the Children's Hospital (Department of Pediatrics) and the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania.

TABLE I

Quantity of Liver Regeneration and New Cytochrome *c* in Liver after 14 Days of Restoration on High (31 Per Cent) Protein Diet, Following Excision of Different Amounts of Liver

In Rats H6 to H9 the left lateral lobe (36.2 per cent of the liver) was excised. In Rats 4 to 18 the left lateral and median lobes (68.4 per cent) were excised.

Rat No.	Body weight		Liver excised		Weight of original total liver	Amount of remaining liver	Cytochrome <i>c</i> in remaining liver†		Final total liver		New or restored liver tissue	Cytochrome <i>c</i> in final liver‡		New cytochrome <i>c</i> in restored liver§		
	Preoperative	Final	Amount	<i>W</i> : <i>D</i> •			Per gm. tissue, wet weight	Total	Amount	<i>W</i> : <i>D</i>		Per gm. tissue, wet weight	Total			
																(4)
(1)	(2)	(3)			(5)	(6)					(9)				(11)	
	gm.	gm.	gm.		gm.	gm.	γ	γ	gm.		per cent	γ	γ	γ	per cent	
H6	268	281	2.94		8.12	5.18	180	933	6.93	3.36	59.5	184	1275	342	26.8	
H7	221	247	2.42	4.01	6.68	4.26	181	771	5.65	3.33	57.4	203	1147	376	32.8	
H8	288	275	2.84	3.43	7.57	4.73	268	1268	5.99	3.22	44.4	265	1588	320	20.2	
H9	332	321	3.11	3.37	8.59	5.48	290	1590	7.51	3.30	65.4	289	2170	580	26.7	
4	286	287	6.72	3.20	9.82	3.10	191	592	7.14	3.19	60.1	214	1528	936	61.4	
5	239	241	5.08	3.18	7.43	2.35	175	411	6.30	3.34	77.8	192	1210	799	66.0	
6	265	257	5.55	3.32	8.11	2.56	168	431	6.21	3.38	65.8	215	1332	901	67.6	
10	248	245	5.13	3.21	7.51	2.38	177	421	5.88	3.28	68.3	208	1222	801	65.6	
11	288	291	5.12	3.24	7.49	2.37	174	412	6.82	3.34	87.0	198	1349	937	69.5	
12	255	252	5.69	3.32	8.33	2.64	200	530	6.24	3.25	63.6	220	1372	842	61.3	
16	174	181	4.23	3.48	6.18	1.95			5.04	3.39	73.0	203	1021			
17	200	209	4.51	3.28	6.02	2.11	172	363	6.44	3.34	96.0	229	1473	1110	74.8	
18	212	231	6.15	3.67	9.00	2.85	171	487	7.36	3.32	73.4	207	1522	1035	74.5	
Mean ± s.e.¶				3.60			230			3.30	56.7	235	1545	405	26.7	
(Rats H6-H9)				±0.20			±31			±0.03		±25				
Rate of appearance of new cytochrome <i>c</i> per day															29	1.9
Mean ± s.e.				3.32			178			3.31	73.9	210	1325	920	67.6	
(Rats 4-18)				±0.05			±4			±0.04		±5				
Rate of appearance of new cytochrome <i>c</i> per day															66	4.8

\* W:D = wet weight to dry weight ratios.

† The values for the concentration of cytochrome *c* per gm. of wet weight of the remaining liver were obtained from the analyses of excised liver. The values for total cytochrome *c* were calculated by multiplying the concentration of the pigment (Column 7) by the corresponding weights of remaining liver tissue (Column 6).

‡ The values for total cytochrome *c* were calculated by multiplying the concentration of the pigment (Column 10) by the corresponding weights of final total liver (Column 8).

§ Total cytochrome *c* in final liver (Column 10) minus the corresponding values for total cytochrome *c* in the remaining liver (Column 7).

¶ From data of Crandall and Drabkin (2).

¶ Standard error =  $\sqrt{2d^2/n(n-1)}$ .



liver was restored in 14 days on the high protein diet in the animals (Rats 4 to 18) in which both the left lateral and median lobes (68.4 per cent of the liver) were excised than in the group (Rats H6 to H9) in which only the left lateral lobe (36.2 per cent of the liver) was removed. The influence of the amount of tissue excised is even more striking in respect to amount and rate of appearance of new cytochrome *c* (Column 11, Table I) than in the amount of regeneration (Column 9). It is seen that, whereas the amount of liver restoration after the excision of two lobes was approximately 33 per cent greater than after the excision of only one lobe (74 and 57 per cent restoration respectively), the amount of appearance of new cytochrome *c* was of the order of 130 per cent greater in the former than in the latter case (920 and 405  $\gamma$ , and 68 and 27 per cent respectively of new cytochrome *c*). It is evident that the removal of approximately two-thirds of the liver afforded a far greater stimulus to new cytochrome *c* production or deposit in the regenerating tissue than did the excision of approximately one-third. In the earlier work of Crandall and Drabkin (2), owing to the small number of animals from which both the left lateral and median lobes were removed, the influence of the amount of tissue excised was not appreciated, and all the results were averaged, yielding a value of 3.5 per cent for the rate of appearance of new cytochrome *c* per day. In the present work (Table I) the rate of appearance of new cytochrome *c* per day (based on a restoration period of 14 days) was only 1.9 per cent after excision of 36.2 per cent of the liver and 4.8 per cent per day after excision of 68.4 per cent of the tissue. When the earlier results (2) after removal of one and two lobes were averaged, there was found a significant increase in the concentration of cytochrome *c* per gm. of wet weight of tissue in the regenerating liver in comparison with the original liver. In this regard the situation is clarified in the present comparison of the effect of the amount of tissue excised. Applying Fisher's criterion (9) for the significance of difference between two means, we find no significant difference in the concentration of cytochrome *c* per gm. of wet weight in the regenerating tissue after the removal of one lobe only and in the original liver. However, following excision of two lobes the concentration of the pigment is significantly greater in the regenerating liver. Here Fisher's value (9) of  $t = 6.5$  and corresponds to a probability,  $P$ , of less than 0.01 that the difference may be attributable to chance. Similar statistically highly significant increases in the concentration of cytochrome *c* in the regenerating liver were obtained in the other experiments (Tables II to IV).

It has been found (7, 10, 11) that the most active cellular proliferation occurs within 4 days after partial hepatectomy in rats. The data given in Table II verify this conclusion and extend it to the appearance of new

cytochrome *c* in liver. The results unequivocally support the inference that on the high protein diet most of the liver regeneration took place and

TABLE II

*Quantity of Liver Regeneration and New Cytochrome c in Liver after 4 and 6 Days of Restoration on High (31 Per Cent) Protein Diet, Following Excision of 68.4 Per Cent of Liver*

Rat No.	Body weight		Liver excised		Weight of original total liver	Amount of remaining liver	Cytochrome <i>c</i> in remaining liver* (7)		Final total liver		New or restored liver tissue	Cytochrome <i>c</i> in final liver* (10)		New cytochrome <i>c</i> in restored liver*	
	Preoperative	Final	Amount	W:D*			Per gm. tissue, wet weight	Total	Amount	W:D		Per gm. tissue, wet weight	Total	γ	γ
(1)	(2)	(3)	Amount	W:D*	(5)	(6)	Per gm. tissue, wet weight	Total	Amount	W:D	(9)	Per gm. tissue, wet weight	Total	γ	per cent
	gm.	gm.	gm.		gm.	gm.	γ	γ	gm.		per cent	γ	γ	γ	per cent
47†	262	263	4.64	3.38	6.78	2.14	175	378	5.24	3.33	67.9	229	1192	814	68.2
48†	228	231	5.55	3.24	8.12	2.57	174	447	5.77	3.33	57.7	216	1247	800	64.2
49†	239	242	4.98	3.28	7.29	2.31	199	459	5.89	3.56	71.7	222	1308	849	65.0
50†	246	248	6.32	3.40	9.25	2.94	192	564	6.56	3.54	57.3	218	1440	876	60.8
51‡	214	216	3.98	3.22	5.82	1.84	171	314	4.82	3.26	74.9	222	1070	756	70.6
52‡	198	202	4.89	3.21	7.15	2.26	183	414	5.10	3.33	58.2	209	1068	654	60.2
53‡	276	277	6.98	3.27	10.21	3.23	177	572	7.48	3.26	60.9	216	1613	1041	64.6
54‡	270	272	6.63	3.26	9.70	3.08	169	521	7.26	3.33	63.2	212	1540	1019	66.1
Mean ± s.e.			3.33				185		3.44	63.6	221	1297	835	64.6	
(Rats 47-50)			±0.04				±6		±0.06		±3				
Rate of appearance of new cytochrome <i>c</i> per day														209	16.1
Mean ± s.e.			3.24				175		3.29	64.3	212	1323	868	65.4	
(Rats 51-54)			±0.02				±3		±0.02		±3				
Rate of appearance of new cytochrome <i>c</i> per day														145	10.9
Mean ± s.e.			3.32				178		3.31	73.9	210	1325	920	67.6	
(Rats 4-18)§			±0.05				±4		±0.04		±5				
Rate of appearance of new cytochrome <i>c</i> per day														66	4.8

\* For methods of calculation and symbols see foot-notes to Table I.

† Regeneration (postoperative) period of 4 days.

‡ Regeneration (postoperative) period of 6 days.

§ Regeneration (postoperative) period of 14 days; from data in Table I.

*all of the new cytochrome c appeared in the first 4 days after liver lobectomy. The rate of appearance of new cytochrome c in liver early after partial hepatectomy was remarkable (Column 11, Table II). The daily incre-*

ments in new pigment were 16.1, 10.9, and 4.8 per cent for regeneration periods of 4, 6, and 14 days respectively. Cytochrome *c* was therefore

TABLE III

*Quantity of Liver Regeneration and New Cytochrome c in Liver after 14 Days Restoration on No Protein Diet, Following Excision of 68.4 Per Cent of Liver*

Rat No.	Body weight		Liver excised		Weight of original total liver	Amount of remaining liver	Cytochrome <i>c</i> in remaining liver*		Final total liver		New or restored liver tissue	Cytochrome <i>c</i> in final liver*		New cytochrome <i>c</i> in restored liver*	
	Preoperative	Final	Amount	W:D*			Per gm. tissue, wet weight	Total	Amount	W:D		Per gm. tissue, wet weight	Total		
	gm.	gm.	gm.		gm.	gm.	γ	γ	gm.		per cent	γ	γ	γ	per cent
1†	203		3.78	3.58	5.54	1.76	146	257							
2	222	187	4.65	3.49	6.78	2.13	133	253	4.87	3.90	59.1	242	1178	925	78.5
3	204	169	3.66	3.46	5.36	1.70	176	299	3.66	3.64	53.5	231	846	547	64.7
7†	198		3.77	3.37	5.52	1.75	128	224							
8	197	154	3.61	3.34	5.29	1.67	176	294	3.62	3.71	54.3	222	806	512	63.5
9†	198		4.35	3.59	6.36	2.01	147	296							
13†	167		3.19	3.96	4.67	1.48	120	178							
14†	168		3.37	3.70	4.92	1.55	124	192							
15	129	105	2.71	3.34	3.96	1.25	144	180	2.85	4.02	59.1	218	622	442	70.1
25	198	163	4.42	3.53	6.47	2.05	140	287	4.35	3.88	51.1	227	987	871	71.1
26	150	123	3.27	3.51	4.78	1.51	152	229	3.33	3.96	55.7	213	709	480	67.8
27	166	135	3.37	3.48	4.93	1.56	165	257	3.39	3.75	54.4	234	794	537	67.6
28	146	121	4.06	3.48	5.94	1.87	134	251	3.35	3.69	36.4	219	734	483	65.9
Mean ± s.e.			3.49				142		3.82		52.9	226 835		588	68.7
(N. P.)‡			±0.04				±4		±0.05			±4			
Rate of appearance of new cytochrome <i>c</i> per day.....														42	4.9
Mean ± s.e.			3.32				178		3.31		73.9	210 1325		920	67.6
(H. P.)§			±0.05				±4		±0.04			±5			
Rate of appearance of new cytochrome <i>c</i> per day.....														66	4.8

\* For methods of calculation and symbols see foot-notes to Table I.

† Did not survive postoperatively. See foot-note 1.

‡ N. P. = no protein diet, Rats 1 to 28, this table.

§ H. P. = high protein diet, Rats 4 to 18, Table I.

produced or deposited early in the process of tissue restoration. It also becomes obvious that calculations of the daily increment in new cytochrome *c* in liver, based upon analytical values obtained after 14 days of regeneration (2), are artificial.

In Table III data are collected in experiments on the no protein diet, and a comparison is afforded of the mean values in these experiments with those on the high protein diet (Rats 4 to 18, Table I). The absence of protein from the diet reduced appreciably the amount of tissue restoration (Column 9, Table III), but did not abolish it. This suggests that building stones for the formation of liver tissue are obtainable from body stores, but optimal restoration cannot be accomplished in the absence of dietary protein. 14 days preoperatively without dietary protein resulted in a significant decrease in the concentration of cytochrome *c* (Column 7, where for the two means  $t = 3.7$ , and  $P =$  less than 0.01). There was also a slight increase in water content of the liver ( $W:D$  ratios, Column 4). It may be noted that during the ensuing 14 day postoperative period on no protein there was nevertheless a statistically highly significant increase in the concentration of cytochrome *c* (compare the mean values of 142 and 226  $\gamma$  per gm. of wet weight, Columns 7 and 10, where  $t = 10.3$ , and  $P =$  less than 0.01). Indeed, this increase in cytochrome *c* concentration in the regenerating liver was more striking on the no protein than on the high protein diet, particularly since a further significant increase in the water content of the liver occurred during restoration without dietary protein ( $W:D$  ratios, Column 8). If the concentration of cytochrome *c* is expressed per gm. of dry weight of tissue, the values for regenerating liver on no protein and on high protein become respectively 878 and 695  $\gamma$ . It may be deduced from these results that the appearance of new cytochrome *c*, a specialized protein, is relatively independent of protein in the diet. That cytochrome *c* is not unique in this connection is, however, evident from the analyses of ribose nucleic acid recorded in Table V.

The data in Table IV were obtained from rats kept on the no protein diet for 14 days preoperatively and for 14 days postoperatively, then shifted to the high protein diet for an additional 14 days. For comparison the mean values are supplied for the expected results if the experiments had been terminated at 14 days after liver lobectomy, as in the case of Rats 1 to 28, on the no protein diet, Table III. The change to the high protein diet resulted in the most remarkable degree of liver restoration which thus far has been obtained after partial hepatectomy either by others (3, 7) or ourselves (2). Regeneration overshot its goal of 100 per cent. When calculated in the usual manner, by Equation 6, total restoration was 170.6 per cent (Column 9), while 117.7 per cent restoration took place after the shift to the high protein. With the alternative method of calculation, based upon the initial body weight before the start of the no protein diet, by Equation 7 (see "Methods"), the total restoration was somewhat lower, namely 138 per cent, but still of extraordinary magnitude. From the mean values in Column 11, Table IV, it is evident that, while a further increase in new cytochrome *c* occurred during the period after

change to the high protein diet, most of the new pigment had appeared in the preceding period of 14 days on no protein. Apparently, in these

TABLE IV

*Effect on Liver Regeneration and New Cytochrome c in Liver by Change from No Protein Diet to High Protein Diet*

The total restoration period was 28 days, 14 days on the no protein diet, followed by 14 days on high protein.

Rat No.	Body weight		Liver excised		Weight of original total liver	Amount of remaining liver	Cytochrome <i>c</i> in remaining liver*		Final total liver		New or restored liver tissue	Cytochrome <i>c</i> in final liver*		New cytochrome <i>c</i> in restored liver*	
	Preoperative	Final	Amount	W:D*			Per gm. tissue, wet weight	Total	Amount	W:D		Per gm. tissue, wet weight	Total		
(1)	(2)	(3)			(5)	(6)					(9)				(11)
	gm.	gm.	gm.		gm.	gm.	γ	γ	gm.		per cent	γ	γ	γ	per cent
19	280	325	4.67	3.57	6.83	2.16	161	348	9.38	3.28	154.8	202	1895	1547	87.6
20	124	151	2.57	3.33	3.74	1.17	156	182	5.04	3.62	150.9	201	1013	831	82.0
21	127	161	2.44	3.51	3.57	1.13	150	169	6.42	3.47	216.5	202	1299	1130	87.2
22	138	193	3.09	3.37	4.53	1.44	174	251	7.41	3.34	193.5	210	1558	1307	84.0
24	127	165	3.23	3.73	4.73	1.50	179	268	5.93	3.37	137.2	192	1139	700	76.6
Mean ± s.e.			3.50				164			3.42	170.6	201	1381	1103	83.5
(Rats 19-24)			±0.07				±5			±0.06		±2			
Rate of appearance of new cytochrome <i>c</i> per day.....														39	3.0
Mean ± s.e.			3.49				142			3.82	52.9	226	835	588	68.7
(Rats 1-28)†			±0.04				±4			±0.05		±4			
Rate of appearance of new cytochrome <i>c</i> per day.....														42	4.9
Difference.....											117.7‡				
												14.8‡			
												1.1§			

\* For methods of calculation and symbols see foot-notes to Table I.

† 14 days restoration on no protein diet; from data in Table III.

‡ Percentile difference between 14 days postoperative restoration on no protein diet and 14 additional days after change to high (31 per cent) protein diet.

§ Daily percentile increment in new cytochrome *c* after change to high protein diet.

experiments conditions were ripe for the utilization of dietary protein in liver regeneration.

In Table V representative results are furnished of analyses for ribose and deoxyribose nucleic acid in liver restoration on the no protein diet (Rats

TABLE V

*Ribose Nucleic Acid (PNA) and Desoxyribose Nucleic Acid (DNA) after 14 Days Restoration,  
Following Excision of 68.4 Per Cent of Liver*

The analyses of PNA and DNA were performed in each case on duplicate aliquots of tissue.

Rat No. (1)	Liver excised (2)		Weight of original total liver (3)	Remaining liver (4)			Final total liver (5)		Nucleic acid in final liver (6)		New or restored liver tissue (7)	New nucleic acid in restored liver† (8)	
	Amount	W:D*		Amount	PNA mg. per gm.†	DNA mg. per gm.†	Amount	W:D	PNA mg. per gm.†	DNA mg. per gm.†		PNA mg.	DNA mg. per cent
64	3.75	3.89	5.48	1.73	9.60	2.36	3.66	3.83	13.10	3.09	51.4	31.3	65.3
65	2.78	3.92	4.07	1.29	10.20	2.73	2.85	3.70	12.50	3.06	56.1	22.4	62.9
66	5.21	3.89	7.62	2.41	9.45	2.58							
67	3.29	3.71	4.82	1.53	9.80	2.75	3.45	3.92	11.20	3.01	58.4	23.6	61.2
68	4.43	3.21	6.48	2.05	8.10	2.44	5.65	3.51	6.80	3.52	81.3	21.8	56.7
69	4.37	3.35	6.39	2.02	8.10	2.50	5.22	3.40	7.55	3.43	73.2	23.0	58.3
70	4.93	3.35	7.21	2.28	9.10	2.47	5.28	3.28	7.10	3.58	60.8	16.7	44.5
71	5.03	3.39	7.35	2.32	8.70	2.41	6.15	3.17	6.40	3.58	76.1	19.2	48.7
Mean ± s.e. (N. P.)§		3.85 ±0.05			9.76 ±0.16	2.60 ±0.09		3.82 ±0.06	12.27 ±0.55	3.05 ±0.03	55.3	25.8	63.1
Mean ± s.e. (H. P.)		3.32 ±0.04			8.50 ±0.25	2.46 ±0.02		3.34 ±0.07	6.96 ±0.24	3.53 ±0.04	72.9	20.2	52.1
												6.2	61.0
												14.4	72.8

\* Symbols as in Table I.

† Calculations for new nucleic acid performed similarly to those for new cytochrome c in Tables I to IV.

‡ Wet weight of tissue.

§ N. P. = no protein diet, Rats 64 to 67.

|| H. P. = high protein diet, Rats 68 to 71.

64 to 67) and on the high protein diet (Rats 68 to 71). The differences in the given mean values for the two groups were all highly significant statistically (9). The quantity of DNA bore a close relationship to the quantity of liver tissue, and it may be seen that the percentile increments in DNA (Column 8) were closely similar to the percentages of restoration of liver (Column 7). This is not surprising since DNA is a constituent of cell nuclei and cellular proliferation has occurred. The changes in PNA, on the other hand, were remarkable. There was an unusually large increase in concentration of PNA during regeneration on the no protein diet. In spite of the higher water content of the liver on the no protein diet (Column 5), there was a 2-fold greater concentration of PNA in the regenerating liver on no protein than on high protein (Column 6). As a consequence of this difference in concentration the total quantity of PNA (calculated from the values in Columns 5 and 6) and the increment in PNA (Column 8) were, indeed, slightly greater on the no protein diet, although regeneration was some 30 per cent less in this case (compare the values in Column 7). It seems clear that the cytoplasmic PNA is involved in some way in the regenerative process and is independent of dietary protein, even more strikingly so than cytochrome *c*, parallel changes in which have been described above. It should be emphasized that sufficient PNA has been formed on no protein; so that if these animals had been shifted to high protein (in experiments comparable to those presented in Table IV) there would be no further production of PNA.

#### DISCUSSION

The various experiments which have been carried out suggest that the concentration and production of certain constituents of tissues, such as cytochrome *c* and ribose nucleic acid, are relatively independent of dietary protein. This appeared to be particularly strongly indicated in the experiments on liver regeneration on a no protein diet, regarded as affording the maximal "deprivation stimulus" for regeneration, in which unusually marked increases in the concentration of both cytochrome *c* and PNA were found in the tissue attempting to regenerate under nutritional conditions far removed from ideal.

Both *intrinsic* (tissue) and *extrinsic* (dietary) factors appear to be concerned in the tissue regenerative process after partial hepatectomy. This was best illustrated in the experiments involving a change from no protein to high protein during the restoration period. After the "deposit" in the liver tissue of intrinsic factors (cytochrome *c* and PNA in the present work) on the no protein diet, allowance of extrinsic dietary protein resulted in unusually great liver regeneration. It may be postulated that the intrinsic factors exert a priming influence and play a rôle in the utilization of dietary protein in restoration. It is of interest that the intrinsic factors

may prove to be constituents of the cytoplasm rather than the nucleus. By means of differential centrifugation cytochrome oxidase, closely associated with cytochrome *c*, has been shown (10, 11) to be a constituent of the mitochondrial or large aggregate fraction of the cytoplasm. PNA is also cytoplasmic but not limited to the mitochondria (11).

On the basis of our experiments the provisional hypothesis is favored that certain cellular, perhaps cytoplasmic structural components, like cytochrome *c* and PNA, are preferentially produced in tissues and are important or essential in normal growth and proliferative processes. The extraordinary increase in the concentration of PNA in tissue regenerating under the stress of nutritional deprivation (absence of dietary protein) suggests that ribose nucleic acid particularly may be involved in the process of protein synthesis. Quantitative chemical evidence for this rôle of PNA does not appear to have been available heretofore, although Caspersson and his colleagues (12) on the basis of histochemical studies have proposed relationships of nucleic acids to cellular protein synthesis.

#### SUMMARY

By means of the technique of partial hepatectomy in the rat, liver regeneration, cytochrome *c* metabolism, and nucleic acids have been investigated with reference to a number of variables: the influence of the amount of tissue excised, the duration of the restoration (postoperative) period, and the effect of dietary protein.

The amount of restoration of liver tissue and quantity of new cytochrome *c* in liver were found to be functions of the amount of tissue removed.

Most of the liver regeneration and all of the new cytochrome *c* "production" occurred in the first 4 to 6 days after partial hepatectomy. The rate of appearance of new cytochrome *c* was found to be 16.1, 10.9, and 4.8 per cent per day for restoration periods of 4, 6, and 14 days respectively.

The appearance of new cytochrome *c* and ribose nucleic acid proved to be relatively independent of dietary protein, and the concentrations of these tissue constituents were, significantly, appreciably greater in regenerating liver from rats on the no protein diet than from those on the high protein diet. The experiments appear to favor the view that PNA may be particularly concerned with protein synthesis during regeneration.

In experiments involving a change during the restoration period from no protein to high protein, unusually great liver regeneration was obtained.

The various experiments have led to the conclusion that certain cellular components, like cytochrome *c* and PNA, are preferentially produced or "deposited" in tissues, and are important or essential in growth and proliferative processes, which appear to depend on *intrinsic* (tissue) as well as *extrinsic* (dietary) factors.



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# LIVER REGENERATION AND CYTOCHROME *c* METABOLISM. INFLUENCE OF ANOXIA AND OF INJECTION OF CYTOCHROME *c*\*

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In a continuation of studies of the effect of various factors on cytochrome *c* metabolism and liver regeneration in partially hepatectomized rats, reported in the preceding paper (2), it was found that the quantity of tissue restoration and rate of appearance of new cytochrome *c* in the regenerating liver were functions of the amount of liver excised and the length of duration of the restoration period. Moreover, it was demonstrated that the appearance of new cytochrome *c* in the regenerating tissue was relatively independent of dietary protein. But, after the "deposit" of cytochrome *c* and presumably other cellular constituents as ribose nucleic acid, which was also determined (2), the allowance of protein in the diet resulted in unusual tissue growth (*cf.* (2), experiments involving the change from no protein to the high protein diet). This interesting and probably significant finding appears to favor the hypothesis that certain cellular, perhaps structural, components like cytochrome *c*, are preferentially produced in tissues and are important or essential components in the processes of normal growth and proliferation.

It was also regarded of interest to investigate the influence of anoxia, produced by oxygen deprivation (life at simulated high altitude), and of injection of cytochrome *c* on liver cytochrome *c* and on the restorative process following partial hepatectomy. This work is reported in the present paper.

In the experiments on anoxia resulting from deprivation of inspired oxygen it was hoped that a degree of tissue anoxia would be produced which might have some effect on cytochrome *c* metabolism. Rats appear

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to be unusually resistant to oxygen deficiency (3) and with the moderate high altitude employed in the present work we were defeated in our objective of demonstrating a possible effect of oxygen want either on cytochrome *c* metabolism or on liver regeneration. However, these experiments had the happy outcome of separating unequivocally what may be regarded as the *primary response* to lack of inspired oxygen, the well recognized response of the oxygen carrier or transport system, hemoglobin and red blood corpuscles, from any postulated *secondary response* which might occur, such as an accommodation of the oxygen utilization mechanism, the cytochrome system. For clarity of interpretation the distinction between the two phases, oxygen supply and oxygen utilization, must be borne in mind. On the other hand, from the metabolic standpoint, the presence of the common hemin configuration in hemoglobin and cytochrome *c* renders the independence of response of these pigments to physiological stimuli of interest.

While the view that tissue cytochromes are produced *in situ* was favored, it was pointed out by Crandall and Drabkin (4) that limitations in the present analytical technique (5) did not permit a decision as to whether the new cytochrome *c* which appeared in the regenerating liver had been produced there *de novo* or whether the pigment may have been deposited in the regenerating tissue after mobilization from a "storehouse" such as skeletal muscle. It was therefore pertinent to test the effect of administering cytochrome *c* in comparatively large amounts before and during active liver proliferation. If preformed cytochrome *c* could be deposited in tissue cells, under the above conditions a maximal opportunity for such a process would be afforded.

### Methods

In these experiments the high protein diet, described in the preceding paper (2), was used. For details as to the rats employed, the alimentation period, the procedure of liver lobectomy, and the analytical techniques reference must be made to the above paper (2), as well as to earlier communications (4, 5).

*Oxygen Deprivation*.—The effect of anoxia, induced by simulated high altitude, was studied in a small low pressure chamber of approximately 120 cu. ft. capacity, with the rats in individual cages. Such animals were kept continuously, except for brief interruptions to remove food 1 day preoperatively and for the surgery, during a period of 11 days (5 preoperative and 6 days postoperative) at a moderately high altitude of  $15,500 \pm 100$  feet.<sup>1</sup> This corresponds to a barometric pressure of 420 mm. of Hg and to an oxygen partial pressure in the inspired gas mixture of

<sup>1</sup> "Ascent" and "descent" were gradual in a time interval of about 1.5 to 2 hours.

88 mm. of Hg or about 55 per cent of the normal at sea level. For 1 day postoperatively glucose was included in the drinking water to circumvent the possible development of hypoglycemia, which has been observed in partially hepatectomized rats exposed acutely to very low barometric pressures, corresponding to altitudes of 32,000 to 47,000 feet (6). No untoward symptoms were observed under our conditions. The animals appeared less active than at sea level, and the impression was gained that they consumed more water and less food. The latter may be reflected in the recorded slight loss of weight (Table I). In these rats, besides cytochrome *c*, the concentration of hemoglobin, erythrocytes, and myoglobin was also measured. The red corpuscle count was carried out in the usual manner, but to insure accuracy in the presence of the pronounced polycythemia a dilution of the blood 3-fold greater than normal was used. Hemoglobin was determined spectrophotometrically as cyanmethemoglobin, with the constant  $\epsilon$  ( $c = 1$  mm per liter,  $d = 1$  cm.)  $= 11.5$  at wave-length  $540\text{ m}\mu$ <sup>2</sup> (7). Myoglobin, after quantitative volumetric extraction (4), was also determined spectrophotometrically as cyanmetmyoglobin, with the constant  $\epsilon$  ( $c = 1$  mm per liter, referred to a molecular weight of 16,450, based on 0.34 per cent of iron,  $d = 1$  cm.)  $= 11.3$  at wave-length  $540\text{ m}\mu$  (8).

*Injection of Cytochrome c*—Cytochrome *c* of high purity and biological activity was injected intraperitoneally daily for a period of 9 days before liver lobectomy and for 5 days postoperatively. To avoid error in interpretation owing to the probable presence of cytochrome *c* in extracellular fluid soon (2 to 4 hours) after injection (Table III), the technique was adopted of administering the last doses of cytochrome *c* 24 hours preoperatively and 24 hours before sacrifice of the animal. Thus, partial hepatectomy was performed 10 days after the start of injections, and the experiment was terminated 6 days after the operation.

The cytochrome *c* employed was prepared from a mixture of horse and beef heart muscle by slight modifications in the Keilin and Hartree method (9), according to Preparation 6 of Drabkin (10), except for the omission of dialysis against water and acetone precipitation.<sup>3</sup> The concentration of the final pigment solution was 10.03 mg. per ml., determined spectrophotometrically as ferrocytochrome *c* after reduction with  $\text{Na}_2\text{S}_2\text{O}_4$ , with the provisional constants  $\epsilon$  ( $c = 1$  mm per liter, referred to a molecular weight of 13,000, based on 0.43 per cent of iron,  $d = 1$  cm.)  $= 26.11$ ,

<sup>2</sup> In the notation  $\epsilon$  ( $c = 1$  mm per liter,  $d = 1$  cm.),  $\epsilon = (1/(c \times d)) \times \log I_0/I$ , where the concentration,  $c$ , is expressed in mm per liter,  $d$ , in cm., the intensity of incident radiation,  $I_0$ , is 1.0, and the intensity of transmitted radiation,  $I$ , is expressed as a fraction of unity.

<sup>3</sup> I am indebted to Dr. H. Tint and Dr. J. Seifter of the Wyeth Institute of Applied Biochemistry, Division of Wyeth, Inc., Philadelphia, for the cytochrome *c*.

7.39, and 15.47 at the wave-lengths 550, 535, and 520  $m\mu$  respectively (10). When corrected for the chloride content,<sup>4</sup> the purity of this preparation was close to theoretical for cytochrome *c* of 0.43 per cent iron content, and was appreciably higher than that of the best preparation reported by the author<sup>4</sup> (10). This is of interest and deserves further study since no resort was made to special methods of purification (11). The biological activity of the cytochrome *c* was high, as tested both by the succinate dehydrogenase (12) and hydroquinone method (13), with pig heart and pig-kidney respectively as a source of the oxidase.<sup>5</sup> •1.5 ml. of the preparation, equivalent to 15 mg. of pure cytochrome *c* (of 0.43 per cent iron content), were administered daily. This quantity is equivalent to the total cytochrome *c* content of the body of rats of this size (250 gm.) (4), and during the period of study the total injections amounted to 210 mg. of the pigment.

In these animals cytochrome *c* was determined not only in the tissues of interest, but also in the urine excreted during the whole period.

### Results

The data presented in Table I show conclusively that the anoxia of oxygen deprivation, induced by life at simulated, moderate high altitude, had in the rat no effect on either liver regeneration or appearance of new cytochrome *c*. On the other hand, data obtained from the same animals on the concentration of hemoglobin and erythrocytes (Table II) indicate that the deficit of oxygen in the inspired gas was of sufficient degree to call forth a rapid and striking response of the oxygen transport system.<sup>6</sup> There appeared to be a greater increase in the number of red blood corpuscles

<sup>4</sup> According to Dr. Tint's analyses, the total dry solids in the preparation were 13.48 mg. per ml., while the solids minus Cl as NaCl were 10.36 mg. per ml., indicating that the solution contained, exclusive of NaCl, cytochrome *c* (of molecular weight 13,000) of 97 per cent purity  $((10.03 \times 100)/10.36)$ . Drabkin's Preparation 6 (10) had a purity of 79.6 per cent. It remains to be established whether the provisional spectrophotometric constants, which were used (see the text) are correct, or whether the present high purity is a matter of chance, or the earlier preparation, which was acetone-precipitated and dried, was of higher purity than reported, owing to the possibility that it may not have been salt-free or thoroughly dry.

<sup>5</sup> The activity was determined by Dr. H. Tint. While total cytochrome *c* concentration is determined most accurately spectrophotometrically, it should be pointed out that the spectrum of ferrocytochrome *c* does not invariably guarantee biological activity. Unpublished data suggest that, whereas the activity of cytochrome *c* solutions, prepared by the Keilin and Hartree procedure (9), is retained practically unchanged when these solutions are kept at refrigerator temperature even for several years, such is not the case with cytochrome *c* prepared as a dry powder by means of acetone precipitation and stored at room temperature. The latter material remains soluble and still gives the characteristic spectrum after reduction with  $\text{Na}_2\text{S}_2\text{O}_4$ .

<sup>6</sup> Hemoglobin and erythrocytes; no change was found in the myoglobin content of skeletal muscle.

TABLE I

*Effect of Oxygen Deprivation\* on Liver Regeneration and New Cytochrome c in Liver after Excision of 68.4 Per Cent of Liver*

The restoration (postoperative) period was 6 days on the high protein diet (2).

Rat No.	Body weight		Liver excised		Weight of original total liver	Amount of remaining liver	Cytochrome c in remaining liver†		Final total liver		New or restored liver tissue	Cytochrome c in final liver†		New cytochrome c in restored liver†	
	Preoperative	Final	Amount	W:D†			Per gm. tissue, wet weight	Total	Amount	W:D		Per gm. tissue, wet weight	Total	(11)	
															(4)
(1)	(2)	(3)			(5)	(6)	γ	γ	gm.		(9)	γ	γ	γ	per cent
35	240	212	6.01	3.39	8.80	2.75	177	487	6.21	3.58	56.9	219	1360	873	64.1
36	228	205	5.72	3.29	8.37	2.65	166	440	6.02	3.74	58.9	208	1251	811	64.9
37	254	231	6.24	3.29	9.12	2.88	165	475	6.88	3.22	63.2	216	1485	1010	68.1
38	239	211	5.54	3.21	8.10	2.57	181	465	5.41	3.72	51.6	197	1065	600	56.4
39	233	232	5.30	3.21	7.76	2.46	179	440	6.28	3.21	60.9	219	1372	932	67.9
40	240	215	4.97	3.07	7.25	2.29	195	446	6.01	3.47	75.0	204	1225	779	63.5
41	236	226	6.38	3.22	9.34	2.96	165	488	5.91	3.03	54.2	237	1400	912	65.3
42	260	242	5.87	3.40	8.60	2.73	152	415	6.03	3.23	48.2	210	1270	855	67.4
43	252	233	5.50	3.18	8.06	2.56	194	497	6.02	3.38	63.0	211	1269	772	60.9
44	263	249	5.67	3.29	8.30	2.63	172	453	7.34	3.41	83.0	210	1540	1087	70.6
45	205	197	3.79	2.96	5.50	1.71	189	323	4.80	3.58	81.5	195	936	613	65.5
46	155	157	4.05	3.60	5.93	1.88	150	282	4.12	3.58	55.3	214	881	599	67.9
Mean ± s.e. (high altitude)‡			3.26 ±0.05				174 ±5			3.43 ±0.06	62.6	212 ±3	1255	820	65.2
Rate of appearance of new cytochrome c per day.....														137	10.9
Mean ± s.e. (sea level controls)§			3.24 ±0.02				175 ±3			3.29 ±0.02	64.3	212 ±3	1323	868	65.4
Rate of appearance of new cytochrome c per day.....														145	10.9

\* Anoxia, induced by simulated high altitude for 5 preoperative and 6 postoperative days (see "Methods").

† For symbols and calculations see foot-notes to Table I, preceding paper (2).

‡ Rats 35 to 46, this table.

§ Rats 51 to 54, Table II, preceding paper (2).

than in the concentration of hemoglobin, particularly during early acclimatization (5 days preoperative). The unequal increase in hemoglobin and erythrocytes was responsible for the deviation from normal of the ratio of the two quantities (Column 4, Table II). The exact significance

of this change in ratio is uncertain, but it may be used as evidence that the increase in the concentration of hemoglobin and erythrocytes was not a consequence of anhydremia. That dehydration was not a factor was also suggested by the state of the tissues at sacrifice, and by the wet to dry weight ratios (Columns 4 and 8, Table I). Indeed, there was a slight

TABLE II

*Mean Values for Hemoglobin and Erythrocyte Concentration in Partially Hepatectomized Rats (Table I) Subjected to Anoxia of Simulated High Altitude*

(1)	Hemoglobin, gm. per 100 ml. blood	Erythrocytes, millions per c.mm. blood	Ratio of hemoglobin to erythrocytes*
(2)	(3)	(4)	
Controls at sea level .....	15.9	7.7	2.06
5 days preoperative at 15,500 ft. ....	21.9	14.9	1.47
6 " postoperative " 15,500 " .....	21.6	11.7	1.85

\* Values in Column 2 divided by corresponding values in Column 3.

TABLE III

*Relationship of Interval of Time after Intraperitoneal Injection of 15 Mg. of Cytochrome c to Analyses of Cytochrome c, C, in Tissues\**

Rat No. (1)	Body weight (2)	Interval after cytochrome c injection (3)	Liver (4)		Kidney cortex (5)		Skeletal muscle (6)	
			W:D†	C	W:D	C	W:D	C
	gm.	hrs.		γ per gm.		γ per gm.		γ per gm.
60	216	0.5	3.23		4.34	680	3.95	112
31	215	2	3.38	191	3.66	692	3.95	119
59	241	2	3.32	200	4.28	670	3.76	102
32	228	4	3.46	203	3.74	440	3.87	111
58	244	4	3.13		4.08	544	4.03	108
33	263	24	3.38	191	4.08	331	3.75	99
34	234	24	3.20	202				
57	216	24	3.17	186	4.14	382	3.83	105

\* For cytochrome c values in normal rats, not injected with cytochrome c, refer to Crandall and Drabkin (4).

† For symbol see foot-notes to Table I, preceding paper (2).

increase in the water content of the regenerating livers of the rats at high altitude (Column 8, Table I).

The analytical results furnished in Table III indicate that transitory increases in the concentration of cytochrome c in tissues may occur following the injection of the pigment. In these experiments such increases in cytochrome c concentration above our previously established normal values

(2, 4) were obtained only in the case of kidney cortex (Column 5). In this tissue there was a very marked rise in cytochrome *c* concentration during the first 2 hours after the injection. At 4 hours the concentration was still elevated, but 24 hours following injection it was essentially

TABLE IV

*Effect of Injection\* of Cytochrome c on Liver Regeneration and New Cytochrome c in Liver After Excision of 68.4 Per Cent of Liver*

The restoration (postoperative) period was 6 days on the high protein diet (2).

Rat No.	Body weight		Liver excised		Weight of original total liver	Amount of remaining liver	Cytochrome <i>c</i> in remaining liver† (7)		Final total liver		New or restored liver tissue	Cytochrome <i>c</i> in restored liver† (10)		New cytochrome <i>c</i> in restored liver†							
	Preoperative	Final	Amount	W:D†			Per gm. tissue, wet weight	Total	Amount	W:D		Per gm. tissue, wet weight	Total								
																(4)		(8)		(9)*	
																(2)	(3)	(5)	(6)	(7)	(8)
(1)	gm.	gm.	gm.		gm.	gm.	γ	γ	gm.		per cent	γ	γ	γ	per cent						
29	233	236	5.31	3.37	7.77	2.46	236	580	7.44	3.46	93.7	205	1522	942	61.8						
30	226	210	5.67	3.55	8.30	2.63	232	610	6.80	3.28	73.7	186	1263	653	51.7						
55	246	242	5.03	3.30	7.36	2.33	188	438	6.22	3.44	77.4	198	1230	792	64.4						
56	240	231	5.07	3.28	7.41	2.34	201	470	6.16	3.54	75.3	193	1190	720	60.5						
Mean ± s.e. (cytochrome <i>c</i> injected)‡				3.38 ±0.06			214 ±12			3.43 ±0.05	80.0	196 ±4	1301	777	59.6						
Rate of appearance of new cytochrome <i>c</i> per day														129	9.9						
Mean ± s.e. (controls)§				3.24 ±0.02			175 ±3			3.29 ±0.02	64.3	212 ±3	1323	868	65.4						
Rate of appearance of new cytochrome <i>c</i> per day														145	10.9						

\* 15 mg. of cytochrome *c* were injected intraperitoneally per rat per day, during a preoperative period of 9 days and a postoperative period of 5 days (see "Methods"). Of the total of 210 mg. injected, 29 mg. or approximately 14 per cent of the cytochrome *c* were eliminated in the urine.

† For symbols and calculations see foot-notes to Table I, preceding paper (2).

‡ Rats 29 to 56, this table.

§ Rats 51 to 54, Table II, preceding paper (2).

normal for kidney cortex (4). It appears reasonable to interpret these changes in cytochrome *c* concentration as being due to the presence of the pigment, following intraperitoneal injection, in progressively decreasing amounts in the extracellular fluids and plasma, rather than as evidence of



the temporary incorporation of the injected pigment in the tissue (14). Some of the injected cytochrome *c* is excreted by the kidneys (see below), in which extracellular fluid (and urine) may be more effectively "temporarily trapped" than in liver or skeletal muscle, thereby accounting for the analytical results.

In Table IV data are given for liver regeneration and cytochrome *c* in the liver of rats on the high protein diet (2), which were injected daily with cytochrome *c* during a period of 10 days preoperatively and 6 days postoperatively. To avoid the transitory changes which could occur early after cytochrome *c* administration (Table III), discussed above, the injection was omitted in the 24 hour periods before liver lobectomy and before termination of the experiment (see "Methods"). Of the total of 210 mg. of cytochrome *c* injected intraperitoneally, 29 mg. or approximately 14 per cent were eliminated in the urine, in which the pigment was determined after reduction of means of direct spectrophotometry.<sup>7</sup> Proger and his colleagues (14) claimed that, under their conditions, following intravenous administration of the pigment, cytochrome *c* was not found in urine.

In these experiments the evidence for the "deposit" of the injected cytochrome *c* in liver is equivocal. The concentration of cytochrome *c* in the liver removed at operation (Column 7, Table IV) was statistically significantly greater than that in the non-injected controls. On the other hand, the incorporation in liver of the injected cytochrome *c* could not be deduced from the values of either the concentration of the pigment or total pigment in the regenerating tissue (Column 10). However, attention is directed to an unexpected finding in these experiments, an approximately 25 per cent greater regeneration of liver tissue in the cytochrome *c*-injected animals (Column 9).

#### DISCUSSION

The interesting outcome of the present experiments on the deprivation of inspired oxygen was the finding that our conditions permitted a clear separation of two variables in anoxia, oxygen supply and oxygen utilization. The conclusion may, therefore, be drawn that the *primary response* to lack of inspired oxygen is the accommodation of the oxygen transport system. The increase in hemoglobin presumably allowed sufficient oxygen to be carried to the tissues; so that no *secondary response* of the

<sup>7</sup> Unpublished experiments in this laboratory show that, when larger amounts of cytochrome *c* are injected intravenously into dogs, fully 50 per cent of the pigment is recoverable in the urine within the 1st hour after its administration, and that in comparison with hemoglobin the urinary "threshold" for cytochrome *c* is low and the urinary "clearance" relatively high.

oxygen utilization mechanism, the cytochrome system, was observed, or, indeed, required.

The observation of increased liver regeneration in rats injected with cytochrome *c* may be significant and important. If this effect is real, it could not be related to an incorporation of the injected pigment in the regenerating tissue, although the high concentration of cytochrome *c* found preoperatively may have a bearing on the increased regeneration. It must also be pointed out that this effect of cytochrome *c* injection may be non-specific, and further experiments are required for a full evaluation of these experimental results.

#### SUMMARY

The effects of anoxia and of cytochrome *c* injection upon liver regeneration and cytochrome *c* in liver were investigated.

Oxygen deprivation by means of simulated, moderate high altitude was without effect on liver regeneration or on cytochrome *c* metabolism, following partial hepatectomy in rats. However, under these anoxic conditions there was a marked increase in the concentrations of hemoglobin and erythrocytes. These findings appear to indicate that the primary response to lack of inspired oxygen is the accommodation of the transport system.

The incorporation of injected cytochrome *c* was not demonstrated unequivocally in the regenerating liver, in which a maximal opportunity for such a process would appear to be afforded.

An appreciable fraction of injected cytochrome *c* was excreted by the kidneys.

In preliminary experiments increased liver regeneration was found in rats injected with cytochrome *c*. Further experiments are required to evaluate this observation fully.

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# THE SYNTHESIS OF GLYCOGEN BY RAT HEART SLICES

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In preliminary studies on the effect of insulin and other hormones upon the synthesis of glycogen from glucose by rat heart slices we used the customary phosphate-saline media with disappointing results. In a search of the literature we found no systematic studies of the factors which influence this synthesis *in vitro*. Accordingly, we began experiments on this problem which are here reported.

**Methods**—White rats were either fed *ad libitum* or fasted for 24 hours. The rats were decapitated, and the heart removed and placed in a beaker containing medium to be used in the experiment. Slices 0.5 mm. thick were made with a Stadie-Riggs (1) tissue slicer, blotted on filter paper, weighed on a torsion balance, and placed in conical Warburg vessels together with 3 ml. of the appropriate medium. The vessels, containing an alkali inset for CO<sub>2</sub> absorption, were then gassed for 3 minutes with 100 per cent O<sub>2</sub> and placed in a water bath at 38° and shaken. After 2 hours, the vessels were removed, and the media and heart slices were transferred to centrifuge tubes and made up to 40 per cent KOH with solid pellets. Glycogen was determined by conventional methods and expressed as micromoles of glucose per wet gm. of tissue. As a rule, the initial glycogen was determined on slices from the same heart and the change of glycogen observed was expressed as  $\Delta$  glycogen. Oxygen uptake was calculated from the manometric readings and expressed as micromoles per gm. The insulin used was an amorphous preparation (Lilly) which assayed 22 units per mg.

**Diabetic Rats**—Rats were made diabetic by fasting for 48 hours and then injecting subcutaneously 200 mg. of alloxan per kilo.

## *Medium for Optimum Synthesis of Glycogen by Rat Heart Slices*

It is our opinion that imitations of "physiological" media for metabolic studies of tissues *in vitro* are somewhat futile. The conditions for metabolism of tissues *in vitro*, particularly with cut tissues such as homogenates or slices, are so different from those *in vivo* that it is impossible to predict in advance what medium is most suitable for the study of a particular re-

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action. This is illustrated by the contrasting experience of Hastings (2) and Stadie and Zapp (3). The former found that an all-potassium medium was best suited for glycogen formation from glucose by rat liver slices, whereas the latter reported that glycogen synthesis by rat diaphragms was far superior in all-sodium medium.

We therefore sought to find a medium most suited to glycogen synthesis by rat heart slices. The components of the medium should not stimulate glycogenolysis, interfere with the determination of glycogen, or be glycogenic.

*Effect of Varying Character of Ions*—Preliminary experiments with six rats showed that glycogen synthesis by heart slices in a medium containing 1 per cent glucose and sodium salts (total osmolarity = 0.3) was quite

TABLE I

*Comparative Effect of Sodium and Potassium Ions upon Glycogen Synthesis by Paired Rat Heart Slices*

Medium, phosphate (sodium or potassium) (pH 6.8) 0.040 M,  $MgCl_2$  0.005 M, glucose 0.011 M, chloride (sodium or potassium) to 0.300 osmolar. Time, 2 hours;  $t = 38^\circ$ .

No. of hearts	Balance of 0.300 osmolar		Oxygen uptake, mean	Final glycogen (as glucose), mean
	Na <sup>+</sup>	K <sup>+</sup>		
	<i>per cent</i>	<i>per cent</i>	<i>micromoles per gm.</i>	<i>micromoles per gm.</i>
6	100	0	148	5.1
	0	100	146	1.3
3	100	0	146	9.3
	50	50	146	1.6
2	100	0	138	11.6
	95	5	160	9.8

small and somewhat dependent on the initial concentration of glycogen. When this was high, glycogenolysis rather than glycogen formation was found. Comparison of the effects of sodium and potassium (Table I) showed that whereas the oxygen uptake was unaffected the final glycogen values were consistently lower in the presence of potassium, indicating possibly an increased glycogenolysis rather than synthesis in the presence of this ion. In this respect heart slices appear to resemble rat diaphragms.

*Effect of Chloride and Phosphate Ions*—The relative effects of phosphate and chloride ions (sodium) upon glycogen formation are given in Table II. Although high phosphate increased oxygen uptake, glycogenolysis rather than glycogenesis occurred. The synthesis with sodium chloride alone was very slight.

*Glycogen Synthesis in Media Containing No or Low Ions*—In all our

experiments glycogen synthesis from glucose (0.1 to 0.3 per cent) in a medium containing sodium salts exclusively was disappointingly small. It was conceivable that the presence of ions of any type is unfavorable to glycogen synthesis. This was first tested by comparing glycogen synthesis in an all-glucose medium (5.4 per cent = 0.3 osmolar) with one containing 1 per cent glucose + sodium salts (Table III). There was found a marked glycogenesis (about 34 micromoles per gm. = 0.6 per cent) upon substitution of ions by glucose. The effect might have been due to the elimination of the ions or the increase in glucose concentration. In

TABLE II

*Comparative Effect of Chloride and Phosphate Ion upon Glycogen Synthesis by Paired Heart Slices from Rats*

Medium, sodium as either chloride 0.123 M or phosphate (pH 6.8) 0.098 M, glucose 0.055 M. Time, 2 hours;  $t = 38^\circ$ . Samples from three rats.

Anion of medium	Oxygen uptake, mean <i>micromoles per gm.</i>	Glycogen (as glucose), mean	
		Initial <i>micromoles per gm.</i>	Final <i>micromoles per gm.</i>
Chloride.....	144	6.1	7.1
Phosphate.....	166	6.1	0.6

TABLE III

*Comparative Glycogen Synthesis by Rat Heart Slices in Phosphate-Saline and Isotonic Glucose Media*

Medium I, sodium phosphate (pH 6.5) 0.040 M,  $\text{MgCl}_2$  0.005 M,  $\text{NaCl}$  0.065 M, glucose 0.055 M; Medium II, glucose 0.300 M. Time, 2 hours;  $t = 38^\circ$ . Samples from two rats.

Medium No.	Oxygen uptake, mean	Final glycogen (as glucose), mean
	<i>micromoles per gm.</i>	<i>micromoles per gm.</i>
I	142	10.6
II	140	34.4

hypotonic glucose solution (0.055 M) no synthesis was found. In addition the oxygen uptake was low and there was marked glycogenolysis (mean, 15 micromoles per gm. per hour).

Since the tissues appeared water-logged, it was obvious that isosmolarity had to be retained. Search was made for a non-ionic substance which could maintain isosmolarity and at the same time be metabolically inactive. Sucrose was eliminated because it was carried over with the glycogen and gave erroneously high values. A series of substances (Table IV) was tried at 0.245 M together with 1 per cent glucose (0.055 M). Of

these substances, L-arabinose, D-sorbitol, and D-mannitol gave satisfactory synthesis of glycogen.

In repeated control experiments none of these substances interfered with the recovery of known amounts of glycogen added to aqueous media with or without tissue.

*Mannitol, Arabinose, or Sorbitol As Glycogen Precursors*—In view of reports in the literature that the intact animal can synthesize glycogen from sorbitol, presumably in the liver, the possibility that this occurs with heart slices *in vitro* was tested. In all experiments with mannitol or arabinose at 0.3 M, glucose being absent, there was glycogenolysis rather than glycogenesis. Fifteen rat hearts were similarly tested with 0.3 M sorbitol. Depending upon the initial glycogen content, there was glycogenolysis

TABLE IV

*Glycogen Synthesis by Rat Heart Slices from Glucose (0.055 M) Medium Made Isosmotic (0.300 Osmolar) by Various Organic Compounds*

Time, 2 hours;  $t = 38^{\circ}$ .

Balance to 0.300 osmolar by	Oxygen uptake, mean*	Initial glycogen (as glucose), mean*	$\Delta$ glycogen (as glucose), mean*
	micromoles per gm.	micromoles per gm.	micromoles per gm.
Sodium chloride-sodium phosphate . . . . .	144	22.0	-5.4
Urea . . . . .	84	22.0	-17.5
Creatine . . . . .	78	10.8	-7.6
Creatinine . . . . .	100	10.8	-8.6
Glycerol . . . . .	100	8.9	+4.7
L-Arabinose . . . . .	138	8.9	+12.5
Sorbitol . . . . .	132	8.1	+11.4
Mannitol . . . . .	118	8.1	+12.7

\* Mean of paired slices.

ranging from 2 to 23 micromoles (as glucose) per gm. per 2 hours. In no case was glycogen formation observed.

*Comparative Effect of Sodium Chloride and Sorbitol on Glycogen Synthesis*—In preliminary experiments the inhibiting action of sodium chloride on glycogen synthesis by rat heart slices was shown by comparison of a 0.055 M glucose solution together with either 0.245 osmolar sodium chloride or sorbitol. In the former case there was glycogenolysis rather than glycogen synthesis, indicating that the sodium chloride either inhibited glycogen synthesis or stimulated glycogenolysis. This was further illustrated by determining glycogen synthesis in a series of paired slices from one rat heart by varying the osmolar proportions of NaCl and sorbitol in the presence of 0.055 M glucose (Table V). The data show that even at this

high concentration of glucose (1 per cent) glycogen synthesis decreased with increasing proportions of NaCl, despite increasing oxygen uptakes.

*Effect of Magnesium upon Glycogen Synthesis*—In enzyme extract systems, magnesium has been shown to be a cofactor in the formation of glycogen from glucose. It was important to determine whether it affected synthesis in heart slices. Paired slices from six rat hearts were

TABLE V

*Effect of Varying Concentration of Sodium Chloride upon Oxygen Uptake and Glycogen Synthesis by Rat Heart Slices*

Medium, glucose 0.055 M, NaCl as in table, sorbitol to 0.300 osmolar. Time, 2 hours;  $t = 38^\circ$ .

No. of rats	NaCl, per cent of total osmolarity	Oxygen uptake, mean	$\Delta$ glycogen (as glucose), mean
		<i>micromoles per gm.</i>	<i>micromoles per gm.</i>
2	0	116	+12.4
2	20	124	+10.8
2	40	128	+7.3
1	60	144	+0.8
2	84	158	+1.2

TABLE VI

*Effect of Magnesium upon Glycogen Synthesis by Paired Heart Slices from Six Rats*  
Time, 2 hours;  $t = 38^\circ$ .

Medium	Oxygen uptake per 2 hrs., mean	$\Delta$ glycogen (as glucose), mean	Effect of ions, mean	
	<i>micromoles per gm.</i>	<i>micromoles per gm.</i>	<i>micromoles per gm.</i>	
Glucose 0.300 M.....	117	9.7	$5.5 \pm 1.3^*$	
MgCl <sub>2</sub> 0.005 M	116	15.2		
KCl 0.005 M				
Glucose to 0.300 osmolar)				

\* Throughout the paper the standard error of the mean (S.E.M.) is calculated by the equation,  $\sqrt{\Sigma d^2 / (n(n-1))}$ , where  $n$  = the number of observations and  $d$  = the difference of observation from the mean.

equilibrated in 0.3 M glucose alone or with 0.005 M MgCl<sub>2</sub> (and 0.005 M KCl). The data (Table VI) show that magnesium has a statistically significant effect in increasing glycogen synthesis. Separate experiments showed that the small amount of KCl (0.005 M) present in this medium was without influence on the synthesis.

*Effect of pH of Medium on Glycogen Synthesis*—This was tested by using a medium containing sodium phosphate 0.010 M, MgCl<sub>2</sub> 0.002 M, glucose



0.017 M, and sorbitol 0.252 M. The glycogen synthesis at pH 6.5, 6.8, and 7.2 showed no significant variation.

In summary, our experience showed that, of the media tested, one which contained a minimum amount of ions was best fitted for the study of glycogen synthesis by rat heart slices *in vitro*. Isosmolarity could be obtained by the addition of a metabolically inert organic compound, the one selected being sorbitol. We thought it advisable to add a low concentration of phosphate for buffering purposes. Our standard medium contained sodium phosphate 0.010 M (pH 6.8 to 7.2),  $\text{MgCl}_2$  0.002 M, glucose (as desired), sorbitol to 0.300 osmolar. Although we had demonstrated that 0.005 M  $\text{MgCl}_2$  significantly increased glycogen synthesis, we were unable to use such a high concentration in the presence of phosphate. Even at 0.002 M  $\text{MgCl}_2$ , precipitation of magnesium phosphate occurs slowly and the final concentration of  $\text{Mg}^{++}$  is indeterminately lower than 0.002 M. This is reflected in the fact that the enhancement of glycogen synthesis by magnesium is lost when 0.002 M  $\text{MgCl}_2$  + 0.010 M phosphate is used. Comparative experiments with paired heart slices from nine rats showed a mean glycogen synthesis of 13.2 micromoles (as glucose) per gm. per 2 hours with this magnesium-sodium phosphate mixture and 16.4 micromoles without magnesium, a difference which was not statistically significant.

*Effect of Glucose Concentration upon Glycogen Synthesis*—This was studied with heart slices from both fed and fasted rats, since they differ markedly in their ability to synthesize glycogen *in vitro*. In the series of fed rats (Fig. 1) there is a definite logarithmic relation between glycogen synthesis and glucose concentration as indicated by the approximate rectilinearity of glycogen synthesis as a function of glucose concentration expressed logarithmically. This is to be expected, since the degree of saturation of enzymes is usually a logarithmic function of the concentration of the substrate. Synthesis was definite at 0.025 per cent glucose and half the maximum rate was obtained at 0.1 per cent. To double the rate an increase of approximately 25-fold in glucose concentration is necessary.

In the series of heart slices from fasted rats the results were quite different (Table VII). As was expected from the experience reported in the literature, the initial glycogen values for heart were high (mean about 25 micromoles (as glucose) per gm.) in contrast to those in the fed series (mean 8.4 micromoles (as glucose) per gm.). In the presence of this initially high glycogen, no further glycogen synthesis was obtained *in vitro* at any concentration of glucose used (0.011 to 0.27 M) except, perhaps, with 0.3 M glucose with no added phosphate or magnesium. On the contrary, there seemed to be a tendency to glycogenolysis. The marked effect of ions is shown again by the virtual disappearance of

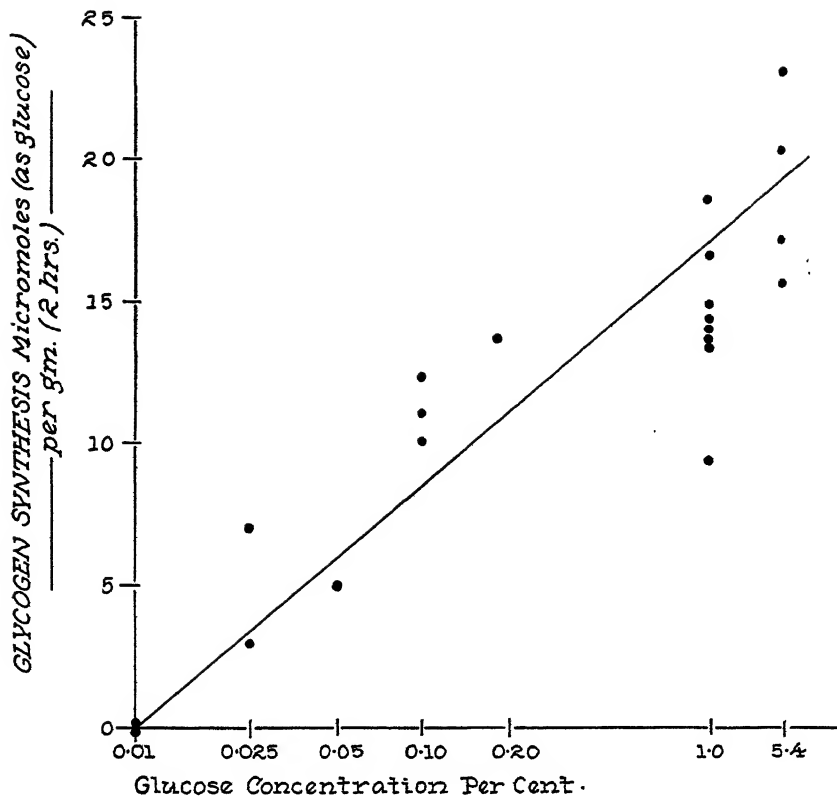


FIG. 1. The synthesis of glycogen by rat heart slices in media containing glucose at varying concentrations with sorbitol to 0.3 osmolar. The concentrations of glucose are logarithmically plotted.

TABLE VII

*Effect of Glucose Concentration upon Glycogen Synthesis by Heart Slices from Fasted (24 Hours) Rats*

Medium,  $\text{MgCl}_2$  0.002 M, sodium phosphate (pH 6.8) 0.010 M, glucose as tabulated, sorbitol to 0.300 osmolar. Time, 2 hours;  $t = 38^\circ$ .

No. of rats	Glucose	$\text{MgCl}_2 + \text{Na}$ phosphate	Oxygen uptake, mean	Initial glycogen (as glucose), mean	$\Delta$ glycogen (as glucose), mean
	M		micromoles per gm.	micromoles per gm.	micromoles per gm.
14	0.30	0	132	22.6	$+6.9 \pm 2.0^*$
3	0.27	+	108	27.2	$-0.5 \pm 2.8$
6	0.055	+	112	24.8	$-6.5 \pm 1.0$
3	0.006	+	110	22.3	$-6.9 \pm 2.8$

\* S.E.M.

glycogen synthesis even at high glucose concentration upon the addition of a comparatively small amount of salts. Such marked effects of ions were not observed with hearts from fed animals. For in this case low salt diminishes but does not eliminate synthesis. The experiments further illustrate the striking difference in the metabolic activity *in vitro* of cardiac and other types of muscle. For example, the diaphragms from rats fed glucose by gavage 4 hours antecedent to the experiment, which contain high initial glycogen, readily synthesize large amounts of extra glycogen *in vitro* (Stadie and Zapp (3)). In the case of heart from fasted rats, likewise containing high initial glycogen, extra synthesis appears not to

TABLE VIII

*Effect of Sodium Lactate upon Glycogen Synthesis of Rat Heart Slices*

Medium,  $\text{MgCl}_2$  0.002 M, sodium phosphate (pH 6.8) 0.010 M, sodium lactate as tabulated, sorbitol to 0.300 osmolar. Time, 2 hours;  $t = 38^\circ$ .

Rat No.	Lactate concentration	Oxygen uptake	Initial glycogen content (as glucose)	$\Delta$ glycogen (as glucose)
	M	micromoles per gm.	micromoles per gm.	micromoles per gm.
1	0.135	200	13.4	-12.8
2	0.050	176	6.7	-1.1
3	0.020	142	17.4	-5.6
4	0.020	140	13.5	-4.7
5	0.020	128	6.3	-0.1
6	0.020	166	4.6	+2.9
7	0.020	156	1.1	+2.9
8	0.020	136	2.7	+4.3
9	0.020	148	9.4	+1.5
10	0.020	160	9.4	+2.1

The values given are the mean of two samples.

occur except in the complete absence of salts and then only with high glucose concentrations in the medium.

*Effect of Substrates Other Than Glucose on Glycogen Synthesis*—At high concentrations of sodium lactate (0.135 and 0.05 M) glycogenolysis occurred *in vitro* (Table VIII). Whether or not this is due to the glycolytic effect of high sodium (from sodium lactate) cannot be said. At low concentrations, when the initial glycogen is low, slight but significant glycogen synthesis occurs.

In twelve experiments with pyruvate (0.015 to 0.15 M) in sorbitol (to 0.3 osmolar) glycogenolysis occurred up to 60 to 100 per cent of the initial glycogen.

No glycogen synthesis was demonstrated in the presence of DL-alanine or  $\beta$ -hydroxybutyrate. As a rule marked glycogenolysis occurred.

*Effect of Insulin on Rat Heart Slices*—This was tested under a variety of conditions in three ways; viz., (1) the effect on glycogen synthesis, (2) the effect on glycogenolysis in absence of glucose in normal rats, and (3) in rats made diabetic by alloxan.

*Effect of Insulin on Glycogenesis*—Unlike diaphragm from fasted or fed rats, in which insulin regularly produces *in vitro* a marked increase in glycogen synthesis from glucose, the effects with rat heart slices were quite variable (Table IX). Heart slices from fed rats with initially low glycogen in 0.3 M glucose showed a very high glycogen synthesis unaffected by insulin. With 0.055 M glucose in sorbitol the mean glycogen synthesis was 12.0 micromoles (as glucose) per gm. and the effect of

TABLE IX

*Effect of Insulin upon Glycogen Synthesis of Rat Heart Slices in Presence of Varying Concentration of Glucose*

Medium, glucose as tabulated, other components to 0.300 osmolar; amorphous insulin, 1 unit per ml. (where indicated). Time, 2 hours;  $t = 38^\circ$ .

No. of rats	Glucose	Other components of medium	Nutrition of rats	Initial glycogen (as glucose), mean	$\Delta$ glycogen without insulin (as glucose), mean	Insulin effect on synthesis (as glucose), mean
	M			micromoles per gm.	micromoles per gm.	micromoles per gm.
9	0.30	0	Fed	10.5	+23.2	+0.5 $\pm$ 1.6*
13	0.30	0	Fasted 24 hrs.	22.2	+6.9	+1.3 $\pm$ 1.7
6	0.055	NaCl + Na phosphate	Fed	6.1	+1.9	+0.3 $\pm$ 1.4
14	0.055	Sorbitol	"	8.1	+12.0	+3.0 $\pm$ 1.1
7	0.006	"	"	7.3	+10.1	+1.6 $\pm$ 1.2
6	0.0014	"	"	11.2	+1.7	+0.3 $\pm$ 0.8
6	0.0006	"	"	9.9	+1.8	+1.5 $\pm$ 1.0

\* S.E.M.

insulin (+3.0  $\pm$  1.1 = 25.0  $\pm$  8.3 per cent) was statistically significant ( $t = 2.7$ ). When the glucose in the medium was low, the glycogen synthesis was also low and insulin had no effect. In fasted rats with initially high glycogen, the glycogen synthesis was also low and insulin had no significant effect.

*Glycogenolysis in Heart Slices from Normal Fasted and Alloxanized Fed Diabetic Rats*—The possibility that insulin might influence glycogenolysis was tested (Table X) with heart slices from normal fasted rats and from rats made diabetic by prior injection of alloxan. The medium contained no glucose. In the normal series the initial cardiac glycogen was high, as is usually the case. Marked glycogenolysis occurred, which

was not affected by insulin. The fed diabetic rats, in contrast to normal fed rats, had a very high initial glycogen. Glycogenolysis was also high and insulin had no effect upon it.

*Glycogen Synthesis in Liver*—The difficulty in generalizing about suitable media for use in studies *in vitro* is illustrated by our experience with liver slices. Unlike muscle, which under suitable conditions *in vitro* will synthesize glycogen at a rate comparable to that achieved *in vivo*, liver slices have not been shown to form glycogen at rates approaching those observed in the intact animal. Our experience with low salt media raised the hope that glycogen synthesis by liver slices could be greatly enhanced. But when the ionic concentration was minimized by the use of isotonic glucose, or glucose + sorbitol, the successful experience with heart slices was not duplicated. Experiments with liver slices from ten rats in various nutritional states were carried out. The medium con-

TABLE X

*Effect of Insulin upon Glycogenolysis of Rat Heart Slices in Medium Containing No Substrate*

Medium, sodium phosphate 0.010 M, MgCl<sub>2</sub> 0.002 M, sorbitol to 0.300 osmolar; amorphous insulin 0.1 unit per ml. (where indicated). Time, 2 hours; *t* = 38°.

No. of rats	Condition of rats	Initial glycogen (as glucose), mean	Δ glycogen (as glucose), mean	Insulin effect (as glucose), mean
		<i>micromoles per gm.</i>	<i>micromoles per gm.</i>	<i>micromoles per gm.</i>
14	Fasted, normal	24.3	-13.0	+2.3 ± 0.9†
6	Fed, diabetic*	34.1	-18.2	+1.0 ± 1.2

\* Made diabetic by injection with alloxan.

† S.E.M.

tained 0.010 M sodium phosphate (pH 6.8), 0.002 M MgCl<sub>2</sub>, 0.001 M CaCl<sub>2</sub>, together with glucose (0 to 0.27 M) and sorbitol up to 0.3 osmolar. The initial glycogen ranged from 0.2 to 42 mg. per gm. The range of oxygen uptake was 94 to 160 micromoles per gm., wet weight, per 2 hours. In no case was there significant glycogen synthesis. On the contrary glycogenolysis occurred as a rule. With liver slices from two alloxanized rats, insulin had no effect either in promoting glycogen synthesis or inhibiting glycogenolysis.

We wish to express our grateful appreciation to Mrs. William McKenna and Mrs. William Tuddenham for technical assistance in the performance of these experiments.

## SUMMARY

1. The synthesis of glycogen from glucose by rat heart slices *in vitro* was studied under a variety of conditions.
2. In a sodium salt medium synthesis is relatively small. With potassium as the cation it is still smaller or does not occur at all.
3. Media containing no or low concentrations of ions are more favorable for glycogen synthesis. Isosmolarity (to 0.3 osmolar) in such media can be obtained by the use of glucose, or sorbitol + glucose, sorbitol itself being non-glycogenic.
4. Magnesium has a statistically significant effect in enhancing glycogen synthesis by rat heart slices. Between 6.5 and 7.2 pH is without influence.
5. The synthesis of glycogen by heart slices is logarithmically related to the glucose concentration. The effect of insulin upon the reaction was also studied.
6. In contrast to heart slices, media with low ionic content do not appear to enhance glycogen synthesis by liver slices.

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# THE EFFECT OF AUTOCLAVING LYSINE IN THE PRESENCE OF CARBOHYDRATE ON ITS UTILIZATION BY THE CHICK\*

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The value of heat treatment in improving the nutritive value of soy bean oil meal has long been known. More recently, however, the deleterious effect of autoclaving too long or at too high a temperature has been demonstrated (1-4). Evans and McGinnis (3) attributed this decrease in nutritive value caused by overheating to the lowered availability of methionine. Riesen *et al.* (5) in work with raw, heated, and overheated soy bean oil meal showed that for the amino acids studied the amount liberated by acid hydrolysis was unaffected by heat treatment, except for lysine, arginine, and tryptophan, and that the liberation of these three was decreased by prolonged heating. Clandinin *et al.* (6) supplemented a practical type of diet containing overheated soy bean oil meal with vitamins, methionine, and lysine, and produced growth comparable to that of chicks receiving properly cooked soy bean oil meal. Thus, overheated soy bean oil meal is apparently deficient in available lysine as well as in methionine.

The lysine in proteins other than soy bean oil meal has been shown to be affected by heat treatment. Harris and Mattill (7) in work with kidney and liver tissue found that hot alcohol extraction produced no loss of nitrogen from the proteins but caused a decrease in the amino nitrogen. To explain this, they suggested the combination of free amino groups with other reactive groups, forming enzyme-resistant linkages. They further suggested that if the free amino groups in proteins are for the most part in lysine, slightly more than half of the  $\epsilon$ -amino groups of lysine disappear during hot alcohol extraction, possibly due to linkage with available carboxyl, imino, or hydroxyl groups. Greaves *et al.* (8), using rats, showed that casein heated for 30 minutes at 140° was deficient in lysine. However, Block *et al.* (9) showed that the same amount of lysine could be isolated, chemically, from raw casein or casein which had been heated for 65 minutes at 150°. Zittle and Eldred (10) confirmed the work of Block *et al.* (9), finding that the lysine was chemically present but not released by enzymes.

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With microbiological techniques it has been demonstrated that a part of the lysine in heated soy bean oil meal was destroyed or unavailable to microorganisms (6).<sup>1</sup>

A reaction has been recently demonstrated between amino acids and reducing sugars. Patton and Pyke (11), working with potato chips, found that the presence of amino acids and reducing sugars produced a browning reaction upon heating. Glycine and glucose produced such a reaction, but so did other combinations of amino acids and reducing sugars. In no case did any sugar or amino acid produce browning when heated alone. Perhaps

TABLE I  
*Composition of Basal Diet*

Ingredients	<i>per cent</i>
Ground corn.....	22.0
" wheat.....	26.4
" barley.....	10.0
Dehydrated alfalfa.....	5.0
B-Y feed (250 $\gamma$ riboflavin per gm.).....	0.5
Ground oyster shell.....	2.0
Dicalcium phosphate.....	1.5
Salt.....	0.5
Soy bean oil meal .....	31.8
Liver paste .....	0.2
Choline chloride.....	0.1
Vitamin D supplement*.....	
MnSO <sub>4</sub> †.....	

\* 100 A. O. A. C. units activated animal sterols were added to each 100 gm. of diet.

† Added at a level of 4 ounces per ton of diet.

there is some similarity between this reaction and the reaction involving lysine in overheated soy bean oil meal or casein.

This study was undertaken to determine whether pure lysine autoclaved alone and in the presence of a carbohydrate could be utilized by the chick as a supplement to overheated soy bean oil meal.

### *Methods*

The soy bean oil meal included in the diets fed in this experiment received one of two treatments. Some diets contained meal of a high nutritive value which had been autoclaved  $\frac{1}{2}$  hour at 100°. The others contained a meal of low nutritive value which had been autoclaved 4 hours at

<sup>1</sup> Evans, R. J., and McGinnis, J., unpublished data (1947).

120°. The color of the soy bean oil meal receiving the prolonged heat treatment was dark brown. There was only a slight change in the color of the meal that was autoclaved  $\frac{1}{2}$  hour at 100°. A diet composed of natural ingredients was fed and its composition is given in Table I.

New Hampshire chicks, not sorted for sex, were used in this experiment. From the time of hatching until 1 week of age they were kept in a battery brooder and fed a complete chick starting ration. At the end of this time they were divided at random into sixteen groups. The chicks were then wing-banded, weighed, and maintained for the following 3 weeks on the experimental diets. Individual weight records were made weekly.

The DL form of lysine<sup>2</sup> and methionine was added to the diets. In the diets in which lysine was used as a supplement, it was added at a level of 0.6 per cent, and when methionine was used it was added at a level of 0.3 per cent. These amounts, in addition to that contained in the basal diet, were sufficient to meet chick requirements, as specified by Almquist and Grau (12). The lysine added to Diet 7 was weighed out into a beaker and autoclaved for 4 hours at 120°. During the heating it changed to a slightly brown color, shrank, and formed a soft cake in the bottom of the beaker. This cake was ground with a mortar and pestle before being added to the diet. The lysine added to Diet 8 was weighed and then mixed in an open tray with "cerelose"<sup>3</sup> (1 part lysine to 10 parts cerelose). The mixture was autoclaved for 4 hours at 120°. The lysine-cerelose mixture formed a black, spongy mass which was mixed with some of the basal diet and ground in a Wiley mill.

#### RESULTS AND DISCUSSION

Table II gives the diets fed, number of chicks per group, and the average weights at 28 days. There was no mortality throughout the experiment. The growth results show that the chicks fed the diet containing soy bean oil meal autoclaved 30 minutes at 100° grew much better than those fed the diet containing soy bean oil meal autoclaved 4 hours at 120°.

Methionine alone had little supplementary value to the overheated soy bean oil meal but, when methionine and lysine were both added, the overcooked meal produced growth nearly equal to that of chicks fed the soy bean oil meal autoclaved for 30 minutes at 100°. Methionine or both methionine and lysine added to the basal diet containing properly heated soy bean oil meal gave only a slight increase in growth.

Autoclaving pure lysine for 4 hours at 120° had no effect on its value as a supplement to overheated soy bean oil meal. On the other hand, autoclaving lysine in the presence of cerelose either destroyed it or made it

<sup>2</sup> The lysine used was in the form of DL-lysine monohydrochloride.

<sup>3</sup> Manufactured by the Corn Products Refining Company, Argo, Illinois.

unavailable. These results indicate that the lysine reacts with some group present in cerelose. Such a reaction might be analogous to the browning reaction of amino acids and reducing sugars suggested by Patton and Pyke (11), as cerelose is composed mainly of glucose. It is interesting to note the similar darkening in color of lysine heated with cerelose and the darkening of overheated soy bean oil meal. In both cases the darkening may have been due to a reaction between the carbohydrate and amino acids present.

TABLE II

*Effect of Autoclaving Lysine in Presence of Carbohydrates on Its Supplementary Value for Overheated Soy Bean Oil Meal*

Diet No.	Autoclaving of soy bean oil meal		Supplements to diet	Initial No. chicks per lot	Average weight at 28 days	
	Time	Temperature			gm.	gm.
	hrs.	°C.				
1	½	100	None	12	238	
				12	222	230*
2	½	100	0.3% DL-methionine	12	246	
				12	246	246
3	½	100	0.3% "	8	229	
			0.6% DL-lysine	8	252	241
4	4	120	None	12	155	
				12	156	156
5	4	120	0.3% DL-methionine	12	164	
				12	173	169
6	4	120	0.3% "	8	222	
			0.6% DL-lysine	8	223	223
7	4	120	0.3% DL-methionine	8	215	
			0.6% treated DL-lysine†	8	234	225
8	4	120	0.3% DL-methionine	8	156	
			0.6% treated DL-lysine‡	8	140	148

\* Average of duplicates.

† Lysine was separately autoclaved for 4 hours at 130°.

‡ Lysine was separately autoclaved as a 1:10 mixture with cerelose, 4 hours at 120°.

It seems probable that the unavailability of lysine in heated casein is due to some other reaction than one with carbohydrate, since casein contains very little carbohydrate. However, it may be possible that some group common to both casein and carbohydrate reacts with the lysine. Assuming that there are two types of reactions, it is possible that they both occur in overheated soy bean oil meal. Even if it is assumed that lysine becomes unavailable on heat treatment due to the formation of an enzyme-resistant linkage with some other substance present, its failure to support the growth

of microorganisms after acid hydrolysis is difficult to explain. Evans and McGinnis<sup>1</sup> after autoclaving soy bean oil meal 1 hour at 130° found that only 70 per cent of the lysine was available on acid hydrolysis for growth of *Streptococcus faecalis* R. Riesen *et al.* (5) found 48 per cent of the lysine available to *Leuconostoc mesenteroides* P-60 after acid hydrolysis of soy bean oil meal that had been autoclaved for 4 hours at 15 pounds pressure. Therefore, it seems that the availability of lysine for bacterial growth is related to the temperature and time of heating. There seems no obvious explanation of this, since lysine is apparently stable to the temperature used in both the above cases.

#### SUMMARY

1. Lysine, autoclaved alone for 4 hours at 120°, supplemented overheated soy bean oil meal for chick growth as effectively as unheated lysine.
2. Lysine autoclaved with cerelose for 4 hours at 120° was either destroyed or rendered unavailable for chick growth.
3. Supplementary methionine and lysine did not improve the growth of chicks fed a diet containing soy bean oil meal which has been autoclaved at 100° for 30 minutes.
4. The decreased nutritive value of *overheated* soy bean oil meal was almost fully corrected by supplementary methionine and lysine.
5. Methionine alone had little supplementary value to overheated soy bean oil meal.

The lysine used in this experiment was kindly provided by Dr. J. Waddell of the E. I. du Pont de Nemours and Company, New Brunswick, New Jersey.

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## LETTERS TO THE EDITORS

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### THE REGRESSION OF LYMPHOSARCOMA IMPLANTS IN PYRIDOXINE-DEFICIENT MICE\*

Sirs:

In a continuation of studies concerning the relation of vitamin B<sub>6</sub> to lymphoid tissue,<sup>1</sup> the administration of a pyridoxine antagonist (desoxy-pyridoxine)<sup>2</sup> was found to produce marked regression on lymphosarcoma implants in mice. Also tumor implants failed to develop in animals deprived of pyridoxine previous to the inoculation.

*Experiment I*—Hybrid mice (mothers of C strain, males C3H) were rendered vitamin B<sub>6</sub>-deficient by feeding a purified diet without added vitamin B<sub>6</sub> and by administration of desoxypyridoxine<sup>3</sup> (300  $\gamma$  per cc.) in the drinking water (Group 2). Controls (Group 1) were maintained on the above diet supplemented with pyridoxine hydrochloride (1 mg. per 100 gm. of diet), and ordinary water. Both groups were inoculated subcutaneously with a mouse lymphosarcoma, 6C3H-ED,<sup>4</sup> after having been on the experimental diet for 3 weeks. 4 weeks after inoculation measurements were taken of three transverse diameters from palpable tumors.

*Experiment II*—The animals were inoculated with lymphosarcoma while on a complete diet. After the implants had grown out, the tumors were measured and the animals (Groups 3 to 6) then placed on the vitamin B<sub>6</sub>-deficient diet and given desoxypyridoxine in the drinking water. A group of controls (Group 7) was given pyridoxine hydrochloride with the diet (20 mg. per 100 gm.) but was otherwise on the same regimen. In some groups the daily water intake was measured. The average consumption of desoxypyridoxine was approximately 900  $\gamma$  per mouse per day. After 5 days on the regimen the tumors were again measured.

The tabulated results show that in most cases implants failed to "take" in animals deprived of pyridoxine. Established tumor transplants were

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<sup>1</sup> Stoerk, H. C., and Zucker, T. F., *Proc. Soc. Exp. Biol. and Med.*, **56**, 151 (1944)  
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<sup>2</sup> Ott, W. H., *Proc. Soc. Exp. Biol. and Med.*, **61**, 125 (1946).

<sup>3</sup> Generously supplied by Merck and Company, Inc.

<sup>4</sup> Donor mouse obtained through the courtesy of Dr. W. U. Gardner.

Group No.	Maximal diameter		No. of mice without palpable tumor	No. of mice with tumors less than 1 cm. across	Total No. of mice
	Average	Range			
	cm.	cm.			
1. Control	2.5	0-6.0	2	2	14
2. Deficient		0-<1	10	2	12

Group No.	Before treatment			After 5 days treatment			No. of mice
	Body weight	Maximal diameter		Body weight	Maximal diameter		
		Average	Range		Average	Range	
	gm.	cm.	cm.	gm.	cm.	cm.	
3. Deficient	29	4.5	2 -6	23	1.2	0-3	5
4. “	28	3.8	3 -5.3	19	2	0.3-3.5	5
5. “	26	2.8	1.6-4	20	1.6	0 -3.2	7
6. “	34	4.3	3 -5.5	25	3.1	1.5-4.5	7
7. Control	30	3.7	2.3-5	33	4.7	3 -6	7

brought to regression by the administration of a pyridoxine antagonist when the animals were maintained on a low vitamin B<sub>6</sub> intake. The anti-vitamin failed to exert this effect when pyridoxine hydrochloride, 20 mg. per 100 gm. of diet, was added to the diet.

Obviously, continued pyridoxine deficiency, although acting more slowly, may ultimately be as fatal as lymphosarcoma. Attempts are therefore being made to find conditions under which the administration of the antivitamin will do the least harm to the host while still exerting an inhibitory effect on tumor growth.

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## ON THE NATURE OF PLASMA IODINE

*Sirs:*

Despite the fact that crystalline thyroxine simulates all of the effects of the thyroid hormone, investigators have hesitated to state categorically that thyroxine is the actual form in which the circulating thyroid hormone exists. Since the minute amount of iodine in plasma makes it very difficult to isolate an iodine-containing compound, we have employed the radioactive isotope of iodine,  $I^{131}$ , to study the nature of the iodine contained in plasma.

Rats were injected with 75 microcuries of carrier-free  $I^{131}$  and plasma was removed 24 hours later. The plasma was extracted three times with 5 volumes of butyl alcohol at room temperature. Clear butyl alcohol extracts were obtained by centrifugation. Plasma  $I^{127}$  was found to pass quantitatively into the butyl alcohol fraction under these conditions. Moreover, only about 10 per cent of this iodine could be reextracted from the butyl alcohol with 4  $N$  NaOH-5 per cent  $Na_2CO_3$ , a reagent which extracts inorganic iodine and diiodotyrosine iodine. These experiments demonstrate that approximately 90 per cent of the plasma iodine of the rat behaves like thyroxine in its solubility properties.

Further identification of plasma iodine with thyroxine was effected by two separate procedures.

25 mg. of three times recrystallized thyroxine (Squibb) were added as carrier to the butyl alcohol extract and the latter was concentrated to dryness on a boiling water bath under reduced pressure. The thyroxine in the residue was repeatedly recrystallized and the specific activity of the  $I^{131}$  determined after each recrystallization. The constancy of the specific activity is shown in Table I. It appears from these results that the  $I^{131}$  in the butyl alcohol fraction of plasma is in the same chemical form as the material which underwent recrystallization. Especially is this true since all recrystallizations were not carried out with the same solvent. The first recrystallization was made from hot 0.1  $N$   $K_2CO_3$  by the addition of glacial acetic acid, the second from a more concentrated 0.1  $N$   $K_2CO_3$  solution by cooling to  $0^\circ$  in an ice bath, the third by addition of glacial acetic acid to a 70 per cent alkaline alcohol solution.

10 mg. of three times recrystallized thyroxine were added as carrier to the butyl alcohol extract and the latter concentrated to dryness, as described above. The thyroxine residue was then dissolved in 0.1  $N$  NaOH and the solution shaken with either butyl alcohol or isoamyl alcohol. The distribution of the  $I^{131}$  between the two immiscible solvents was compared with that of the  $I^{127}$  (Table II). Within experimental error it was found



TABLE I

	Experiment 1	Experiment 2	Experiment 3
	<i>counts per min. per <math>\gamma</math> I</i>	<i>counts per min. per <math>\gamma</math> I</i>	<i>counts per min. per <math>\gamma</math> I</i>
Initial.....	2.64	2.57	2.90
After 1st recrystallization.....	2.58	2.25	2.69
“ 2nd “ .....	2.58	2.20	2.81
“ 3rd “ .....	2.47	2.28	2.74
“ 4th “ .....	2.53	2.28	2.70

TABLE II

Experiment No.	Solvent pair	$\gamma$ I per cc.	I <sup>131</sup> per cc.	Specific activity $\times 10^3$
			<i>counts per sec.</i>	<i>counts per sec. per <math>\gamma</math> I</i>
1	0.1 N NaOH	100	9.7	9.7
	Butyl alcohol	167	17.3	10.3
2	0.1 N NaOH	98.2	10.0	10.2
	Butyl alcohol	170	17.3	10.2
3	0.1 N NaOH	264	25.3	9.6
	Isoamyl alcohol	18.2	1.83	10.0

that the distribution of the I<sup>131</sup> was the same as that of the I<sup>127</sup> carrier.

The evidence presented here, we believe, is the strongest yet put forth for the view that the circulating thyroid hormone is actually thyroxine.

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# INCORPORATION OF LABELED GLYCINE INTO THE PROTEIN OF TISSUE HOMOGENATES\*

Sirs:

The incorporation of labeled methionine into liver slices<sup>1</sup> and of glycine into intestinal slices<sup>2</sup> has been reported. However, homogenates of these tissues were inactive.

The present experiments demonstrate that, under suitable conditions, homogenates can take up labeled amino acid. The medium employed was similar to that suggested by Cohen and McGilvery<sup>3</sup> in their study of *p*-aminohippuric acid formation by homogenates. It had the following composition: 100 ml. of 0.9 per cent NaCl, 8 ml. of 1.1 per cent KCl, 1 ml. of 1.2 per cent KH<sub>2</sub>PO<sub>4</sub>, 2 ml. of 3.8 per cent MgSO<sub>4</sub>·7H<sub>2</sub>O, 21 ml. of 1.3 per cent NaHCO<sub>3</sub> (saturated with CO<sub>2</sub>), 100 mg. of glucose, 50 mg. of

Tissue	Treatment	Counts per mg. protein per min.	
		Active homogenate	Homogenate boiled prior to incubation
Spleen.....	Homogenized in presence of glycine	1.6	0.2
	Glycine added after homogenizing	2.2	0.1
	Medium replaced by distilled water; homogenized in presence of glycine	0.1	0.0
Liver.....	Homogenized in presence of glycine	2.6	0.1
Intestines.....	“ “	0.02	0.01

sodium citrate, 25 mg. of fumaric acid, 20 mg. of adenosine triphosphate, 20 mg. of coenzyme I, and 10 mg. of cytochrome c. Calcium was not included. The solution was oxygenated and adjusted to pH 7.5.

5 ml. of medium, approximately 0.25 gm. of homogenized rat tissue, and 2 mg. of radioactive glycine (labeled on the  $\alpha$ -carbon with C<sup>14</sup>) were incubated at 36° for 1.5 hours.<sup>4</sup> Then trichloroacetic acid was added and the proteins were prepared as previously described.<sup>2</sup> The results are given in the table.

\* Aided by grants from the American Cancer Society (recommended by the Committee on Growth of the National Research Council) and the Rockefeller Foundation.

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<sup>4</sup> The glycine had an activity of approximately 200,000 counts per mg. per minute.

Both spleen and liver homogenates took up glycine upon incubation to the extent of about 0.1 per cent of the amounts employed in the media. The heat-inactivated controls had almost no activity. Homogenates of intestines showed no significant uptake. The spleen showed approximately the same activity when the labeled glycine was added either before or after homogenizing. It is significant that spleen homogenates were virtually inactive when the nutrient medium was replaced by distilled water. The importance of the individual components of the medium, as well as the various factors which regulate the rate of amino acid uptake, is being investigated.

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## THE POSITION OF STREPOGENIN IN THE PROTEIN MOLECULE

Sirs:

Since the structure of strepogenin, a peptide-like growth factor constituting an integral part of several proteins,<sup>1</sup> is not yet known, evidence for its position in the protein molecule is of interest. A free amino group is essential for strepogenin activity.<sup>1, 2</sup> If this amino group is free in the protein, then treatment of the intact protein with reagents which act on amino groups should result in products which do not give rise to strepogenin when digested with trypsin or with acid. On the other hand, if the strepogenin is not at the end of the peptide chain of the protein, then such treatment should not alter the strepogenin potency.

Reaction of insulin with dinitrofluorobenzene almost completely destroyed its strepogenin potency as measured with *Lactobacillus casei*.<sup>1</sup> Sanger<sup>3</sup> has shown that this reagent reacts under very mild conditions with the free amino groups of insulin. Before assay for strepogenin the dinitrophenyl insulin was digested with trypsin in one series of experiments. To avoid error which might arise from inability of the enzyme to attack the altered protein and liberate the growth factor, tests were also made on hydrolysates obtained by allowing DNP-insulin to stand in concentrated HCl at room temperature for 3 hours. This manner of hydrolyzing proteins has been shown to yield values for strepogenin in casein and insulin which are one-fourth those found with trypsin digestion. However, treatment of DNP-insulin with concentrated HCl produced no strepogenin.

Similarly, insulin deaminated by HNO<sub>2</sub>, when digested either with trypsin or with concentrated HCl, gave less than 10 per cent of the strepogenin yielded by the unaltered protein.

Sanger<sup>3</sup> has shown that the free amino groups of insulin are due solely to glycine, phenylalanine, and lysine. The inactivation of strepogenin in insulin by dinitrofluorobenzene or HNO<sub>2</sub> would thus implicate these three acids in the strepogenin molecule. However, since concentrates of the growth factor have been obtained free of detectable lysine and phenylalanine, it seems probable that glycine supplies the free amino group of strepogenin. The recent observations of Sanger<sup>4</sup> have allowed us to gain more evidence for this view. He showed that oxidation of insulin yielded a

<sup>1</sup> Woolley, D. W., and Sprince, H., *Federation Proc.*, **4**, 164 (1945). Sprince, H., and Woolley, D. W., *J. Am. Chem. Soc.*, **67**, 1734 (1945).

<sup>2</sup> Woolley, D. W., Symposium on body proteins, University of Buffalo (1946).

<sup>3</sup> Sanger, F., *Biochem. J.*, **39**, 507 (1945).

<sup>4</sup> Sanger, F., *Nature*, **160**, 295 (1947).

peptide in which the free amino groups were due entirely to glycine, and a second fraction in which the lysine and "end" phenylalanine resided. We have prepared these fractions and found only the former to contain strepogenin.

The tentative conclusions which these data seem to justify are that in insulin the strepogenin structure is at the end of the peptide chain, that some of the free amino groups of the protein belong to the growth factor, and that glycine is the amino end-group of strepogenin. If dinitrofluorobenzene and  $\text{HNO}_2$  react with groups other than  $-\text{NH}_2$  in insulin, these conclusions may be in jeopardy.

Trypsinogen and casein, two other proteins rich in strepogenin, have been examined similarly. In trypsinogen, as in insulin, potency was destroyed by alteration of the amino groups. In casein, however, about half was retained after such procedures.

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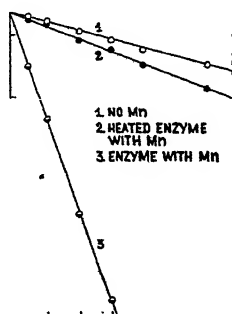
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## THE FIXATION OF CARBON DIOXIDE IN A PLANT TRICARBOXYLIC ACID SYSTEM\*

Sirs:

A preparation from parsley root has previously been shown to contain a reversible oxalacetic carboxylase and a malic dehydrogenase, and to be capable of fixing  $\text{CO}_2$  in dicarboxylic acids.<sup>1</sup> Evidence is presented here that this same parsley root preparation contains an oxalosuccinic carboxylase and an isocitric dehydrogenase, and is capable of fixing  $\text{CO}_2$  in the tricarboxylic acids.



Decarboxylation of oxalosuccinic acid by parsley root preparation. Experimental conditions: 7.8 mg. of parsley root protein in total volume of 2.0 ml.; 0.125 M acetate buffer, pH 5.5, and  $5 \times 10^{-4}$  M  $\text{MnCl}_2$ . Oxalosuccinic acid, kindly supplied by Dr. S. Ochoa, tipped in from side arm to start reaction.  $\text{CO}_2$  evolution measured manometrically at  $20^\circ$  under anaerobic conditions.

The evidence for the presence of oxalosuccinic carboxylase is shown in the figure. The reaction is of the first order with respect to substrate and requires  $\text{Mn}^{++}$ .

The evidence for the presence of isocitric dehydrogenase was obtained by measuring reduction of TPN spectrophotometrically at 340  $\text{m}\mu$ . The reduced TPN could be reoxidized by  $\alpha$ -ketoglutarate and bicarbonate. DPN was not reduced by isocitrate.

When isocitrate and  $\alpha$ -ketoglutarate were incubated with the parsley root enzyme,  $\text{Mn}^{++}$ , TPN, ATP, and  $\text{NaHC}^{14}\text{O}_3$ , fixation of  $\text{C}^{14}$  occurred in the isocitrate fraction.  $\alpha$ -Ketoglutarate isolated as the 2,4-dinitrophenyl-

\* Aided in part by grants from the John and Mary R. Markle Foundation and from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

<sup>1</sup> Gollub, M. C., and Vennesland, B., *J. Biol. Chem.*, **169**, 233 (1947).

hydrazone contained no  $C^{14}$ . The table shows that addition of TPN without ATP gave some fixation, that the omission of (1) TPN, (2) ATP and TPN, or (3)  $\alpha$ -ketoglutarate and isocitrate resulted in no fixation, but that ATP added to TPN enhanced the quantity of  $C^{14}$  fixed.

Incubation mixture*	$C^{14}$ fixed in organic acid fraction†
Complete system*	100
Omit ATP	27

\* 0.02 M phosphate buffer, pH 6.7,  $2.7 \times 10^{-3}$  M  $MnCl_2$ , 0.033 M  $\alpha$ -ketoglutarate, 0.0067 M DL-isocitrate, 0.003 M  $NaHC^{14}O_3$ , and  $2 \times 10^{-4}$  M ATP. 71 mg. of lyophilized parsley root protein and 100  $\gamma$  of TPN added to total volume of 15 ml. Incubated at 30° for 3 hours.

† Radioactivity of ether extracts expressed as relative values. 100 is equivalent to a total fixation of about 3 per cent of the  $C^{14}$  added.

These experiments support the hypothesis that tricarboxylic acids may be formed in plants by a process involving the addition of  $CO_2$  to  $\alpha$ -ketoglutarate, in a manner similar to that already demonstrated in animal tissues by Ochoa.<sup>2</sup>

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<sup>2</sup> Ochoa, S., *J. Biol. Chem.*, **159**, 243 (1945). Ochoa, S., and Weisz-Tabori, E., *J. Biol. Chem.*, **159**, 245 (1945).

# THE CONDUCTANCE OF SOLUTIONS OF SODIUM BENZYL PENICILLINATE

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(Received for publication, August 16, 1947)

The conductance of solutions of penicillin salts is a governing factor in iontophoretic treatment of infections with this antibiotic. In contrast to the experiments of von Sallmann and Meyer (1) showing an increased transport of penicillin to the aqueous humor of rabbit eyes under a D.C. potential of 45 volts, Hamilton-Paterson (2) states that "penicillin appears to be either a very poor conductor or a non-conductor."

This conclusion was based on current measurements in extremely dilute ( $\approx 25$  to 50 units per ml.) sodium penicillin solutions with equipment not designed to investigate ion mobilities. A high D.C. voltage (240 volts) maintained over a period of 1 to 2 hours undoubtedly produced electrolysis of water with attendant pH changes at the electrodes, which in turn were responsible for the observed indicator color changes and for the reported destruction of penicillin, a substance known to be very unstable (3) above pH 10 and below pH 4. Furthermore, the increase in current to be expected from addition of  $\approx 10^{-5}$  to  $10^{-4}$  M sodium penicillin was too small (though significant in Hamilton-Paterson's Experiment 1) to be measured with any certainty in the cell employed, which doubtless had a very high cell constant.

It seems inadmissible, despite the experiments cited, that a sodium salt of penicillin should have a negligible conductance, although it is altogether reasonable to expect the penicillin anion to have a lower mobility than most inorganic or small organic anions. This expectation was confirmed by the conductance measurements of aqueous solutions of crystalline sodium benzylpenicillinate (penicillin G) described in this paper.

## Materials

*Sodium Benzylpenicillinate (Sodium Penicillin G)*—Three moisture-free Merck preparations of crystalline salt were employed in these measurements. Tests presented in Table I indicate the penicillin purity and predominance of the benzylpenicillin form. Assays for penicillin G content by the N-ethylpiperidine (4) method and for total penicillins by the iodometric (5) method, as well as rotations and potency (6) determinations, were performed by the Chemical Control Division of Merck and Company.



Inc. The reported extinction coefficients are corrected<sup>1</sup> for absorption by traces of penicillenate and penamaldate present in solution. The elementary analyses were performed in the Microanalytical Laboratory. Clearly, the penicillin preparations are at least 95 to 96 per cent in the benzyl (G) form, with small amounts of other penicillins present. Solutions ranging in concentration from  $\approx 0.0007$  N to  $\approx 0.048$  N were prepared by direct weighing of the sodium salt into small tared beakers or platinum boats. All conductance measurements were made immediately after preparation of the penicillin solutions.

*Potassium Chloride*—Reagent grade potassium chloride, used for determining cell constants, was fused prior to preparation of a standard 0.01000

TABLE I  
*Properties of Preparations*

	Lot 17	Lot 36	Lot 37
Specific rotation (Na D line), <i>degrees</i>	294.1	291.7	288.8
Penicillin G content (N-ethylpiperidine method), %	94.8*	95.8	95.6
Total penicillins (iodometric assay), %	100.0	104.0	100.4
Potency, <i>units per mg.</i>	1536†	1556	1539
Molecular extinction coefficient (2570 Å)	253	250	249
Carbon content (calculated 53.92%), %	53.63	53.78	53.77
Hydrogen content (calculated 4.81%), %	5.23	5.28	4.68
Nitrogen content (calculated 7.86%), %	7.82	7.82	7.84
Sodium content (calculated 6.45%), %	6.55	6.36	6.49

\* Food and Drug Administration value 94.2 per cent.

† Food and Drug Administration value 1642.

demal (7) solution. This solution contained 0.37312<sub>6</sub> gm. of KCl and 500.000 gm. of water (brass weights, in air).

*Water*—The water employed throughout these experiments was first demineralized by passage through an ion exchanger, and then further distilled through a block tin condenser. No attempt was made to aerate this water. Its specific conductance ran about  $2$  to  $5 \times 10^{-6}$  (ohm<sup>-1</sup> cm.<sup>-1</sup>) and was always measured before salt solutions were prepared. The appropriate water correction was applied in computing the conductance of salt solutions.

<sup>1</sup> Recommended by Dr. N. R. Trenner of this laboratory. A full description will be included in the forthcoming monograph entitled, *Chemistry of penicillin*, being published by Princeton University Press under the auspices of the National Academy of Sciences.

### *Apparatus*

Resistances of solutions were measured by a standard Kohlrausch alternating current bridge (8, 9) arrangement. A Kohlrausch type helical slide wire drum (Leeds and Northrup No. 4258) served as the dividing arms of the bridge; an (Leeds and Northrup No. 4746) a.c.-wound decade box, providing up to 11,110 ohms with an accuracy of  $\pm 0.05$  per cent, served as the third arm; and the bridge was completed by the conductance cell containing the solutions being investigated. Additional Leeds and Northrup (Nos. 4631 and 4640) a.c.-wound fixed resistors were available if needed for water conductance observation in units of 1000 or 10,000 ohms  $\pm 0.1$  per cent. Capacitance effects caused by the cell were balanced by a 0.001 microfarad variable condenser shunted across the decade box, which permitted operation at a power factor of unity.

The bridge was balanced visually by means of a DuMont 208B cathode ray oscillograph (10) which was more convenient than earphones and had the additional advantage of permitting the operator to observe the balancing out of capacitance effects. To achieve maximum accuracy, decade box resistances were chosen to permit a balance of the drum within 1 unit of the 500 mark, which was the exact mid-point of the drum scale.

High frequency alternating current was supplied by an RCA No. 154 beat frequency oscillator, operating on a 115 volt a.c. line and capable of supplying sinusoidal current in frequencies up to 15,000 cycles per second. The operating frequency in these measurements was 1000 to 1500 cycles per second. An output impedance of 250 ohms appeared to be optimum for the bridge circuit. To avoid excessive electrostatic and inductive effects from extraneous sources, the bridge was grounded at the drum contact. Numerous trials attested to the reliability of this arrangement.

Three Pyrex glass cells (designated Cells F, G, and H) of the Jones (11) type, modified to permit greater ease of flushing, and of reduced capacity ( $\approx 5$  ml.), were used to cover the resistance range of the benzylpenicillinate salt solutions; a fourth cell ( $\approx 35$  ml.) of the Washburn (12) design (referred to as Cell A) was reserved for measurements of water conductivities. Electrodes were platinized (13) by electrolysis of a chloroplatinic acid solution containing lead acetate, followed by electrolytic dechlorination. Cells were flushed at least eight times before measurement, and successive observations were made to ascertain constancy of resistance values. Cells were kept immersed in a water thermostat maintained at  $30.05^\circ \pm 0.03^\circ$ . In all solutions studied, temperature equilibrium was established rapidly owing to the small volumes involved; after some 5 to 10 minutes, resistances remained essentially constant. Penicillin solutions appeared to be stable for at least 1.5 hours, which was the normal duration of a measurement,

and in many cases, observations were continued for 3 hours without resistance variation.

TABLE II  
Cell Constants (*J*)

Cell	<i>J</i>
A	0.0287 <sub>6</sub>
F	0.2078
G	0.5573
H	3.474

TABLE III  
Conductance of Sodium Benzylpenicillinate

Penicillin lot No. (1)	Cell (2)	Concentration (3)	$\sqrt{C}$ (4)	Solution, <i>R</i> (5)	$\Delta$ (6)	$\Delta_0$ (7)
		<i>moles per l.</i>		<i>ohms</i>	<i>ohm<sup>-1</sup> cm.<sup>2</sup></i>	
36	F	0.0007015	0.02649	3727.0	76.18	78.46
36	G	0.0007015	0.02649	9992.0	76.21	78.40
17	"	0.001403	0.03746	5132.0	75.20	78.79
17	"	0.002806	0.05297	2634.7	74.27	79.47
17	"	0.005612	0.07491	1348.9	73.08	79.47
17	"	0.005612	0.07491	8413.4	73.04	80.01
17	"	0.008418	0.09175	5660.0	72.16	79.85
17	"	0.009119	0.09550	5258.0	71.76	79.85
36	"	0.009119	0.09550	5290.0	71.67	79.96
37	"	0.009119	0.09550	5298.0	71.57	80.87
17	"	0.009821	0.09910	4904.1	71.48	80.98
17	"	0.01740	0.1319	2857.7	69.55	80.98
17	"	0.01824	0.1351	2730.0	69.47	80.93
36	"	0.01824	0.1351	2742.2	69.30	81.89
37	"	0.01824	0.1351	2740.0	69.36	82.79
17	"	0.01908	0.1381	2624.2	69.08	83.36
17	"	0.02736	0.1654	1872.6	67.66	83.48
17	"	0.03648	0.1910	1434.0	66.32	83.76
17	"	0.04349	0.2086	1220.0	65.34	
17	"	0.04560	0.2135	1169.1	65.05	
36	"	0.04560	0.2135	1171.4	65.01	
37	"	0.04560	0.2135	1170.0	65.07	
17	"	0.04770	0.2184	1120.4	64.88	

Cell constants (*J*), measured by determining resistances of the standard KCl solutions, are listed in Table II. The specific conductance of the 0.01000 demal (7) KCl at 30.05° was taken as 0.001547<sub>0</sub> ohm<sup>-1</sup> cm.<sup>-1</sup>

### Results

Pertinent data and calculations based thereon are compiled in Table III. Columns 1 to 4 are self-explanatory. In Column 5 are found the resist-

ances ( $R$ ) of the solutions calculated from the decade box resistance  $r$  (not listed), and the bridge reading  $x$  (not listed) by means of the standard formula

$$R = r \frac{1000.0 - x}{x} \quad (1)$$

The specific conductance  $\kappa$  (not shown in Table III) is calculated for each concentration by the expression

$$\kappa = \frac{J}{R} \quad (2)$$

and from this quantity is computed the equivalent conductance  $\Lambda$  (Column 6), which is defined as  $1000\kappa/C$ , where  $C$  is the normality (equal to molarity in this case).

Although most of the penicillin salt measurements were made with Lot 17, a large number of concentration levels were repeated with Lots 36 and 37. Several of these check runs are reported in Table III, together with the average value of equivalent conductance. Judging from the mean deviations for these groups, the experiments yield  $\Lambda$  values reproducible to  $\leq 0.1$  unit.

#### DISCUSSION

Inspection of Table III fails to reveal the abnormally low conductance of sodium benzylpenicillinate solutions predicted by Hamilton-Paterson (2), but serves rather to demonstrate the normal electrolyte behavior of the salt. A plot of these data in the form of a  $\Lambda$  versus  $\sqrt{C}$  curve displays the usual curvature (14)<sup>2</sup> typical of strong electrolytes, which makes difficult an accurate extrapolation to infinite dilution. The indication from low concentration points is that the equivalent conductance of this salt at infinite dilution ( $\Lambda_0$ ) is  $>78$ .

A more precise evaluation of  $\Lambda_0$  was attempted by applying the Shedlovsky (15) modification of the Onsager equation (16) for uni-univalent electrolytes, which may be written

$$\Lambda'_0 = \frac{\Lambda + \sigma\sqrt{C}}{1 - \theta\sqrt{C}} = \Lambda_0 + BC \quad (3)$$

All undefined quantities in this equation are constants, and one would expect a plot of  $\Lambda'_0$  versus concentration to be a straight line, with slope equal to  $B$  and a  $\Lambda'_0$  intercept of  $\Lambda_0$ . Values of  $\sigma$  and  $\theta$ , which are functions of dielectric constant and temperature, are found from tables in Harned and Owen (17) to be 67.22 and 0.2299, respectively, for water at 30.05°.

<sup>2</sup> See (9), chapter 18.

Column 7 of Table III contains  $\Delta'_0$  values computed as specified in Equation 3.

Fig. 1 is a plot of  $\Delta'_0$  versus  $C \times 1000$  from which  $\Delta_0$  is usually obtained by extrapolating to zero concentration. A slight curvature (Curve I) is still evident, especially at the low concentrations. This may be due to the failure of theory to account for the unusual size and interactions of an ion such as the benzylpenicillinate ion, or it may result from inaccuracies in  $\Delta$  measurements in the very dilute solutions, because of adsorption errors or the large corrections (up to  $\approx 6$  per cent) for water conductance. In any event, the discrepancy entailed is not serious, since the low concentration points lead to a  $\Delta_0$  of 78.3 and the higher concentration points, con-

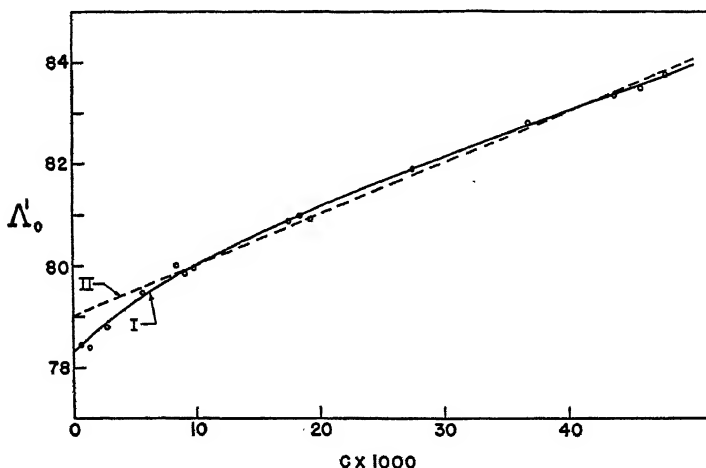


FIG. 1. Variation of  $\Delta'_0$  with concentration of sodium benzylpenicillinate

sidered linear (Curve II), extrapolate to a  $\Delta_0$  of 79.0.<sup>3</sup> If the mobility<sup>4</sup> of the  $\text{Na}^+$  ion at  $30.05^\circ$  is taken as 55.0, and a value of 79 adopted for the equivalent conductance of sodium benzylpenicillinate at infinite dilution, a mobility of 24 is derived for the benzylpenicillinate anion. A mobility of this magnitude is not without precedent among large organic anions (19), and confirms the expected normal electrolyte nature of sodium benzylpenicillinate. The measured mobility of 24 leads to transference numbers of 0.30 and 0.70 for anion and cation, respectively.

<sup>3</sup> Evaluated by the method of least squares by Mr. J. H. Davidson, Chemical Control Division.

<sup>4</sup> This mobility is estimated from  $\text{Na}^+$  ion mobilities at  $0^\circ$ ,  $18^\circ$ , and  $25^\circ$  (18). The temperature coefficient  $\Delta$  at  $25^\circ$  is computed to be 0.0192.

## SUMMARY

1. The conductance of aqueous solutions of sodium benzylpenicillinate (sodium penicillin G) has been measured over a range of concentrations at  $30.05 \pm 0.03^\circ$ .

2. The salt behaves as a completely dissociated electrolyte of the 1:1 valence type, with possible deviations due to ion size and interactions.

3. The limiting equivalent conductance of the salt is  $\approx 79 \text{ ohm}^{-1} \text{ cm.}^2$ , and the mobility of the benzylpenicillinate anion  $\approx 24 \text{ ohm}^{-1} \text{ cm.}^2$ . This relatively low value is reasonable for a large organic anion.

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## INCREASED LIVER PHOSPHATASE ACTIVITY IN ALLOXAN-DIABETIC RATS\*

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Contributions from Cori's laboratory by Colowick *et al.* (3, 4) and Sutherland (5) have proposed the concept that the effects of certain hormones (from the anterior pituitary, adrenal cortex, and pancreas) in carbohydrate metabolism are linked with their action on specific tissue enzymes. The action of insulin as thus far uncovered by the above investigators is not simple, and involves in skeletal muscle the removal of inhibitions to the activity of hexokinase (3, 4), while in liver glycogenesis may be stimulated, presumably through an increase in phosphorylase activity (5). It seems probable that insulin may have still other points of action (3). The present investigation of another of the enzymes prominently involved in carbohydrate metabolism, a study of liver phosphatases in an experimental diabetic state, was encouraged by (1) our findings (6) that alimentary hyperglycemia in rats induces increases in the activities of the acid and alkaline phosphatases of the kidney, and that an *in vitro* inhibition of phosphorylation in kidney homogenates may be produced by the addition of phosphatase, and (2) the report of Thannhauser and his colleagues (7) that in glycogen disease the alkaline phosphatase activity of the liver is diminished. The work has disclosed that in a well established alloxan-diabetic state in rats the activities of both acid and alkaline liver phosphatases are significantly increased. Furthermore, our experiments suggest that in such alloxan-diabetic animals, treated effectively with insulin, there is a return towards normal levels of liver phosphatase activity.

### Methods

Diabetes was induced in male albino rats, 125 to 225 gm. in weight, by intraperitoneal or more usually subcutaneous injection in aqueous solution of 175 to 200 mg. of alloxan monohydrate per kilo of body weight, following a fasting period of 24 to 48 hours, as suggested by Kass and Waisbren (8). Food, Purina dog chow checkers, and water were then allowed *ad*

\* A preliminary report upon this work has been made (1). Simultaneously with the publication of our note, Cantor, Tuba, and Capsey (2) published a preliminary report of studies on serum phosphatase in alloxan-diabetic rats.



*libitum*, with the rats kept in individual metabolism cages, permitting collection of urine not contaminated by food. Convenient criteria of the progress of the diabetic state are the presence of polydipsia and the excretion of abnormally large volumes (25 to 70 ml. per day) of glycosuric urine. At appropriate times after administration of the diabetogenic dose of alloxan the animals were sacrificed by decapitation, and blood and liver samples were taken. Blood sugar was determined by the micromethod of Folin and Malmros (9), with a photoelectric filter photometer for measurement (6). The liver samples were accurately weighed and then homogenized with distilled water for 2 minutes at room temperature in a test-tube homogenizer of the Potter-Elvehjem (10) type. The amount of water used in the homogenization was varied with the weight of sample to maintain a constant concentration of 80 mg. of tissue per ml. of homogenate. Freezing of the tissue in liquid air prior to homogenization, as was done in the previous experiments on kidney tissue (6), was dispensed with, since we have now found that this step in the procedure (6) does not increase the phosphatase activities of liver and kidney homogenates.

Measurement of the acid and alkaline phosphatase activities of the liver homogenates was performed in the same manner as that of the kidney homogenates reported by Marsh and Drabkin (6), with the sodium  $\beta$ -glycerophosphate buffers described by Shinowara, Jones, and Reinhart (11), adjusted to pH 5.0 and 9.3.

In rats in which diabetes was well established (4 days after alloxan administration), treatment with insulin was carried out in two ways. In *Procedure 1*, 0.4 of a unit of regular insulin was administered subcutaneously at intervals of 3 hours for a total of three injections, according to the procedure of Cantor *et al.* (2). The blood sugar and liver phosphatase activities were determined 1 hour following the last dose of insulin. In *Procedure 2*, designed to afford more adequate treatment, insulin was administered for 3 days, beginning 4 days after alloxan. 0.8 of a unit of regular insulin was injected subcutaneously at 3.5 hour intervals for a total of 2.4 units and 1.6 units of protamine zinc insulin were given at the time of the last dose (in the late afternoon or early evening) of regular insulin. The homogenates from the liver samples in these experiments were divided into two aliquots, to one of which 0.5 of a unit of regular insulin was added to test the effect of further addition of insulin *in vitro* upon phosphatase activity.

#### EXPERIMENTAL

In Table I data are presented upon the acid and alkaline phosphatase activities of liver tissue from normal rats. In the alloxan diabetes experiments the animals are fed, but their state of "alimentation" in compari-

son with normal animals is not known. It was, therefore, thought advisable to compare the liver phosphatase activities of normal fed rats and rats fasted 24 hours. In our data (Table I) no significant differences in the mean values for the activities of the liver phosphatases were found in

TABLE I  
*Acid and Alkaline Phosphatase Activities per Gm., Wet Weight, of Liver Tissue from Normal Rats*

Rat No.	State	Blood sugar  <i>mg. per 100 ml.</i>	Ratio, wet to dry weight	Phosphatase activity*	
				pH 5.0  <i>mg. P per hr.</i>	pH 9.3  <i>mg. P per hr.</i>
1	Normal (fed)	105	3.30	7.27	1.31
2	" "	100		8.60	1.76
3	" "	92		6.38	1.27
4	" "	110		7.34	1.15
5	" "	104	3.24	8.76	1.25
6	" "	99		7.93	1.25
7	" "	104		8.66	1.16
Mean $\pm$ s.e.†		102	3.27	7.85 $\pm$ 0.34	1.31 $\pm$ 0.08
8	Normal (fasted†)	71		6.92	1.26
9	" "		3.32	6.85	1.28
10	" "	76		7.20	1.34
11	" "	68	3.28	8.40	1.09
Mean $\pm$ s.e.		72	3.30	7.34 $\pm$ 0.36	1.24 $\pm$ 0.05
Fed and fasted, mean $\pm$ s.e....		93	3.29	7.66 $\pm$ 0.25	1.28 $\pm$ 0.05
12	Normal (fed)			6.38§	1.37

\* Expressed as mg. of P liberated in 1 hour from sodium  $\beta$ -glycerophosphate at 37.5°. This is equivalent to Bodansky units (12).

† Standard error =  $\pm \sqrt{\Sigma d^2/n(n-1)}$ .

‡ Fasted for 24 hours.

§ The acid phosphatase analysis on this animal was run at pH 4.5 to serve as a control for acid phosphatase values reported on Rats 24 to 26 in Table III.

the fed and 24 hour-fasted animals. This finding is not in accord with that of Oppenheimer and Flock (13), who reported a substantial decrease in alkaline phosphatase activity in liver following 24 to 48 hour fasting periods. However, for reasons which are not clear, the normal phosphatase values reported by the above authors both for rat liver and serum are only 25 to 50 per cent of the values found by us and others (1, 2).

The results upon alloxan-diabetic animals are collected in Table II. The data may be analyzed into two groups. In one group, designated "early diabetic," of three animals (Rats 13 to 15), which were sacrificed within 2 days after alloxan administration, no significant change (in comparison with the normal rats, Nos. 1 to 11, Table I) in the liver phosphatase activities was found. One of these animals (Rat 13) had marked liver and kidney damage, and in this rat both the acid and alkaline phos-

TABLE II  
*Acid and Alkaline Phosphatase Activities per Gm., Wet Weight, of Liver Tissue from Alloxan-Diabetic Rats*

Rat No.	State	Period after alloxan	Blood sugar	Ratio, wet to dry weight	Phosphatase activity*	
					pH 5.0	pH 9.3
		days	mg. per 100 ml.		mg. P. per hr.	mg. P per hr.
13	Early diabetic (fed)	1.0			6.44	1.39
14	" " "	2.0	670	3.48	7.74	1.16
15	" " "	2.0	620		6.40	1.46
Mean $\pm$ S.E. ....		1.7	645	3.48	6.86 $\pm$ 0.44	1.34 $\pm$ 0.09
16	Established diabetic (fed)	5.0	700		10.40	1.92
17	" " "	5.0	336		10.30	1.51
18	" " "	7.0	490		10.30	1.47
19	" " "	4.0	411	3.26	10.85	2.02
20	" " "	14.0	262	3.19	7.53	1.92
21	" " "	4.0	550		7.65	1.64
22	" " "	4.0	328	3.36	9.30	1.93
23	" " "	4.0	370	3.32	9.17	1.70
Mean $\pm$ S.E.† .....		5.9	431	3.28	9.44 $\pm$ 0.45	1.76 $\pm$ 0.07

\* See corresponding foot-note to Table I.

† The values for *t* (Fisher (14)) for the difference between the means of the eleven normal rats (reported in Table I) and the eight animals with established diabetes (Rats 16 to 23, this table) are, for the acid and alkaline phosphatase activities, 3.68 and 5.45 respectively. These values correspond in each case to a probability, *P*, of less than 0.01 that these differences may be attributable to chance.

phatase activities of the kidney were appreciably lower than normal for our animals (6). This may be interpreted as a toxic effect of alloxan, which has been reported previously by Menten and Janouch (15). In a second group of eight animals (Rats 16 to 23), the rats were sacrificed 4 to 14 days (average 5.9 days) after the administration of alloxan. In this group, described as "established diabetic," the individuals of which excreted 25 to 70 ml. of glycosuric urine per day, the acid and alkaline phosphatase activities of the liver were increased by 23 and 38 per cent, respectively,

as calculated from the means in these experiments (Table II) and the corresponding mean values for the eleven normal animals (Table I). These increases in liver phosphatase activity in established alloxan diabetes are highly significant statistically, judged by the criterion of Fisher (14) for the significance of difference in two means (*t* and corresponding *P* values in the foot-note to Table II). If these fed diabetic animals may

TABLE III

*Acid and Alkaline Phosphatase Activities per Gm., Wet Weight, of Liver Tissue from Alloxan-Diabetic Rats (Established Diabetics, Fed) Treated with Insulin*

Rat No.	Period after alloxan	Blood sugar	Ratio, wet to dry weight	Phosphatase activity*	
				pH 5.0	pH 9.3
	days	mg. per 100 ml.		mg. P per hr.	mg. P per hr.
27†	4.0	100		9.65	1.94
28†	4.0			10.50	1.82
29†	4.0	262			2.16
Mean ± S.E. ....	4.0	181		10.08 ± 0.43	1.97 ± 0.10
24‡	7.0	57		6.54§ (6.60)	1.23 (1.30)
25‡	7.0	148		6.38§	1.69
26‡	7.0		3.29	6.10§	1.49
Mean ± S.E. ....	7.0	103		6.34 ± 0.13	1.47 ± 0.13

\* See corresponding foot-note to Table I.

† Insulin administered according to Procedure 1, 4 days after the injection of the diabetogenic dose of alloxan.

‡ Insulin administered according to Procedure 2, 4 days after the injection of the diabetogenic dose of alloxan.

§ These acid phosphatase analyses were run at pH 4.5, and the values are to be compared with that of the normal rat, No. 12, Table I.

|| The values in parentheses obtained on aliquot were of the liver homogenate to which 0.5 unit of insulin was added *in vitro*.

be regarded as, in some respects, comparable to normal fasted animals, and if fasting has a tendency to decrease liver phosphatase activity ((13) and see above), the increase in liver phosphatase activity was demonstrated in spite of the possible presence of this effect, operating in the opposite direction.

Attention may be called to the fact that no significant changes were encountered in the ratios of wet to dry weight of the liver tissue of normal and diabetic animals (Tables I and II).

Data obtained in a limited number of experiments on the effect of treatment of established alloxan diabetes with insulin are reported in Table III. Here again, the results fall into the categories of non-success and

success in respect to return of liver phosphatase activity from the elevated diabetic level towards normal. In Rats 27 to 29 insulin was administered according to Procedure 1 (see "Methods"), which Cantor *et al.* (2) recently reported to be effective in lowering an elevated alkaline blood phosphatase activity in their alloxan-diabetic rats. In our animals the liver phosphatase activities remained elevated following this form of insulin therapy, although the blood sugar was lowered. On the other hand, insulin administration according to Procedure 2 (see "Methods") did prove effective in restoring to normal levels (see Table I) both acid and alkaline liver phosphatase activities of rats (Nos. 24 to 26, Table III) with established alloxan diabetes. It may be seen also (Rat 24, Table III) that no greater effect on phosphatase activity could be demonstrated by the further addition of insulin *in vitro* to liver homogenates from alloxanized rats, treated with insulin by Procedure 2.

#### DISCUSSION

A relationship has now been shown to exist between the level of blood sugar and the phosphatase activities of the kidney (6), the blood serum (2), and the liver ((1, 7), and this paper). The present findings that the activities of the liver phosphatases are increased in rats with well established alloxan diabetes, and that they may be restored to normal by effective insulin therapy, suggest that the phosphatases have a significant rôle in the diabetic state.

A comparison of results on serum and liver phosphatase activities after alloxan administration, secured independently by Cantor *et al.* (2) and ourselves (1), is of interest. In the experiments of the former workers (2) serum phosphatase activity was followed. There was found a decrease of activity in the first 6 hours after alloxan, followed by a return to normal in 12 hours, then an elevation above normal by the 2nd day, and in 4 days the activity was double its normal value, with further gradual increases in the ensuing 2 to 3 weeks. In our work (reported in detail here) increases in the activity of the liver phosphatases were not observed until the 4th day after the injection of alloxan. This parallelism of change in phosphatase activity in the two sets of experiments suggests a possible relationship between the phosphatases of liver and serum in the rat. Various tissues have been suggested as main sources of alkaline plasma phosphatase (16), including the liver (13), but at present it appears premature to ascribe to the latter organ a dominant function in this connection. For one thing, there is the disturbing matter, with no explanation, of the relative activities of the acid and alkaline phosphatases respectively of different rat tissues: serum 0.032, 1.13 units<sup>1</sup> per ml. (2), kidney 5.30,

<sup>1</sup> Bodansky units (12). See appropriate foot-note to Table I.

48.1 units per gm. of wet weight (6), and liver 7.66, 1.28 units per gm. of wet weight (our values). Per gm. of tissue the alkaline phosphatase activity of the kidney is some 37-fold greater than that of the liver. The activity of the liver acid phosphatase is 6-fold greater than that of the alkaline phosphatase of this tissue and strikingly greater than that of the acid phosphatase of serum, and yet the changes in serum phosphatase activity after alloxan reported by Cantor and his colleagues (2) were marked only in the case of the alkaline enzyme. On the other hand, in our various experiments on both kidney (6) and liver (1) phosphatases we have consistently observed parallel changes in acid and alkaline phosphatase activities. While the contribution of the liver and other tissues in regard to plasma phosphatase remains to be clarified, this organ is involved in the homeostatic control of the level of blood sugar and probably of plasma inorganic phosphate. Mention may be made of the recognized increase in plasma inorganic phosphate in diabetic coma and the reported relationship in alloxan-diabetic coma (17) of the fall in acid phosphate in liver to the rise in plasma inorganic phosphate, phenomena with which phosphatase activity may be associated.

The demonstration of changes in the activity of tissue phosphatases under such stimuli as hyperglycemia (Marsh and Drabkin (6)) and in such states as alloxan diabetes (present work) invites explanations as to how such changes in tissue enzyme activity are accomplished. It is possible that the enzyme activity is "controlled." Experiments are contemplated to test whether the control is of hormonal character, which, indeed, has already been suggested in experiments on adrenalectomized (18) and castrated animals (19). Hormonal control of the dephosphorylating systems (phosphatases) appears to be probable owing to the demonstration in Cori's laboratory (3, 4) of such control in the functionally closely allied mechanism of phosphorylation (the hexokinase system).

#### SUMMARY

1. Statistically highly significant increases of 23 and 38 per cent respectively in the activities of the acid and alkaline liver phosphatases have been demonstrated to occur in rats with well established alloxan diabetes.

2. Effective insulin therapy applied to rats with well established alloxan diabetes has been found to restore both acid and alkaline liver phosphatase activities to normal levels.

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## THE AMINO ACID COMPOSITION OF BOVINE SEMEN\*

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Very few data are available concerning the amino acid content of mammalian sperm. The early work by Kossel (1) on the protamines of fish sperm and on the histones was primarily concerned with an attempt to elucidate the structure of the basic proteins. The basic proteins are, in general, characterized by a high arginine content. Zittle and O'Dell (2) have determined the methionine content of whole bovine spermatozoa and found an average value of 1.92 per cent (moisture- and fat-free basis). Since the literature is obviously incomplete with respect to the amino acid content of sperm and since some of the problems of infertility might conceivably be related to one or more of the chemical constituents of semen, it seemed advisable to initiate a study of the amino acid composition of bovine semen.

### EXPERIMENTAL

*Preparation of Semen for Analysis*—The amino acid values reported in this paper represent a composite of 149 semen samples obtained from forty different bulls. These bulls were used routinely for artificial insemination and were of the Holstein, Guernsey, and Jersey breeds. All samples were collected by means of the artificial vagina. As the samples were collected they were preserved by freezing at  $-10^{\circ}$  in a stoppered container until a total volume of 250 ml. was obtained. At this time the composite sample was removed from the freezer, thawed, and centrifuged at 5000 R.P.M. for 30 minutes. The supernatant liquid or seminal plasma was decanted, the cells washed once with water, centrifuged, and the wash liquid discarded. The sperm and seminal plasma were again frozen and then dried in this state by sublimation, and the resulting products were white amorphous powders. The seminal plasma dried in this manner contained 1.4 per cent dry matter, while the sperm contained 20 per cent dry matter. Further drying *in vacuo* for 48 hours at  $60^{\circ}$  over phosphorus pentoxide showed that the freeze-dried seminal plasma contained 0.76 per cent moisture while the

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sperm contained 6.80 per cent moisture. These dried products were used for the determination of eleven amino acids.

*Methods*—The amino acid determinations were carried out microbiologically with the organisms *Lactobacillus arabinosus*, *Streptococcus faecalis*, and *Leuconostoc mesenteroides*. The media used in the various determinations are essentially the same as those described by Sauberlich and Baumann (3). The samples were prepared for assay according to the method given by Stokes *et al.* (4) with the exception of the samples for tryptophan, which were prepared according to the method of Wooley and Sebrell (5). Media for stock cultures as well as the inoculum were prepared according to Kuiken *et al.* (6). Determinations were made in triplicate and all values were checked to within  $\pm 5$  per cent. Amino acid determinations were

TABLE I  
*Amino Acid Composition of Sperm and Seminal Plasma\**

Amino acid	Sperm	Seminal plasma
	<i>per cent</i>	<i>per cent</i>
Arginine.....	25.47	7.91
Histidine.....	2.54	2.13
Lysine.....	5.08	4.86
Tryptophan.....	1.59	2.63
Phenylalanine.....	3.81	3.42
Methionine.....	1.81	1.61
Threonine.....	3.78	3.20
Leucine.....	5.20	3.81
Isoleucine.....	3.42	2.79
Valine.....	3.73	3.11
Glutamic acid.....	8.33	7.75

\*All the values are expressed on the moisture-, ash-, and fat-free basis.

also carried out with a sample of purified casein and these values checked with those in the literature (7).

### Results

The sperm and seminal plasma contained 17.61 and 12.05 per cent nitrogen respectively (Kjeldahl) when corrected for moisture, fat, and ash. No attempt was made to convert the nitrogen values to percentage of the protein because of the possibility of the presence of non-protein nitrogenous compounds. However, one possible compound, spermine, could not be detected in the semen of the bull (8). Both the dried sperm and seminal plasma are high in protein.

The amino acid composition of sperm and seminal plasma is shown in Table I. The compositions of the two dried materials are quite similar

with the exception of arginine, leucine, and tryptophan. As was expected, the arginine content of sperm was found to be very high, whereas the concentration of this amino acid in seminal plasma was relatively lower, although the proportion exceeded that of any other of the amino acids studied with the possible exception of glutamic acid. Dried seminal plasma contained a higher concentration of tryptophan than sperm. Methionine is relatively low in both sperm and seminal plasma, a characteristic of many animal and plant proteins. The amino acid distribution in the proteins of sperm and seminal plasma is unusual, as is also the case with such specialized proteins as the keratins and hemoglobins (7).

#### SUMMARY

The concentration of eleven amino acids in bovine sperm and seminal plasma is reported.

With the exception of arginine, leucine, and tryptophan, the amino acid composition of sperm and seminal plasma is quite similar. The arginine content of sperm is very high but is relatively much lower in seminal plasma. The tryptophan concentration in seminal plasma is considerably higher than that found in sperm.

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# NOMOGRAM FOR CORRECTION OF LOW URINE CHLORIDE VALUES DETERMINED BY THE SILVER IODATE REACTION

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Sendroy (1-3) pointed out in his original papers on the determination of chloride by the reaction,  $\text{AgIO}_3 + \text{Cl}^- \rightleftharpoons \text{AgCl} + \text{IO}_3^-$ , that the  $\text{IO}_3^-$  formed reduces the  $\text{AgIO}_3$  solubility to a negligible value in analyses of plasma, and of urines except those with unusually little chloride. Sendroy ((1) Fig. 2) gave a curve of corrections for  $\text{AgIO}_3$  solubility at 20-25°, for use in analyses of low chloride urines. The temperature solubility curves of Van Slyke and Hiller ((4) Fig. 1) afford a basis for estimating the corrections at temperatures varying over the maximal range of laboratory conditions. In accurate measurements of chloride balances of subjects with minimal excretions it has been found that, even when the 5-fold increased samples of urine recommended for such cases (4) are used, the corrections can become significant, especially with high summer temperatures.

The corrections are calculated by Equation 16 of Sendroy (1), viz.

$$(1) \quad [\text{Cl}]_i = 1.0043[\text{IO}_3^-]_e - \frac{K_{\text{AgIO}_3}}{[\text{IO}_3^-]_i}$$

$[\text{Cl}]_i$  is the chloride concentration of the solution that is shaken with  $\text{AgIO}_3$ ;  $[\text{IO}_3^-]_e$  is the dissolved  $\text{IO}_3^-$  concentration at the end of the reaction between  $\text{AgIO}_3$  and  $\text{Cl}^-$ ; and 0.0043 is the ratio  $K_{\text{AgCl}} \cdot K_{\text{AgIO}_3}$ , where  $K_{\text{AgCl}}$  and  $K_{\text{AgIO}_3}$  are the millimolar solubility coefficients of  $\text{AgCl}$  and  $\text{AgIO}_3$  respectively. The ratio of the solubility coefficients remains near enough to 0.0043 over the range of room temperature to make the temperature effect negligible on the factor 1.0043. However, the effect of temperature on  $K_{\text{AgIO}_3}$  is such that the solubility correction, represented by the term,  $-K_{\text{AgIO}_3}/[\text{IO}_3^-]_e$  in Equation 1, can be affected significantly by temperature variations within the range of laboratory conditions.

The corrections indicated by Fig. 1 are calculated by Equation 1 from  $[\text{IO}_3^-]$  values corresponding to the titration figures of the scale on the left side of the nomogram, and from the solubility values of  $\text{AgIO}_3$  in 0.15 M  $\text{H}_3\text{PO}_4$  found by Van Slyke and Hiller (4). Analyses of dilute chloride solutions have verified the accuracy of the theoretically calculated corrections.

The nomogram is designed for direct application to the titrimetric modi-

fication of the chloride method recently described by Van Slyke and Hiller (4), in which the  $\text{IO}_3^-$  in the filtrate from the reaction mixture is titrated with 0.02303 N thiosulfate. A straight line connecting the observed titration value in the left-hand scale with the point on the temperature scale, that represents the temperature at which the  $\text{AgIO}_3$  and chloride solution are shaken, cuts the inner scale at the point indicating the correction to subtract

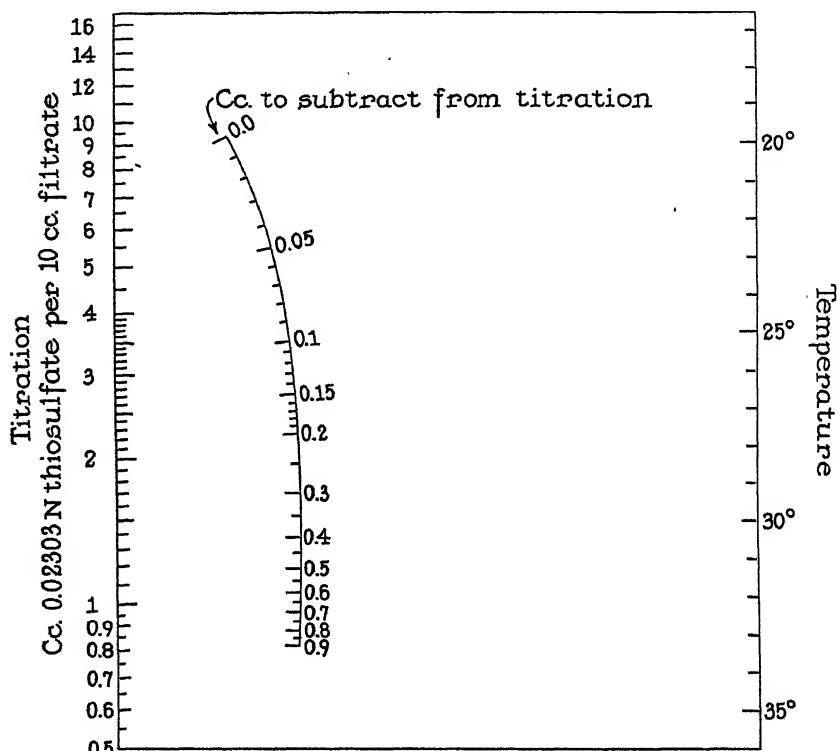


FIG. 1

from the observed titration figure. The corrected titration value is used to calculate urine chloride by the usual formulae (4).

In the form given in Fig. 1, the nomogram is applicable independently of the number of times the urine or other analyzed chloride solution is diluted before it is shaken with  $\text{AgIO}_3$ .

If the line connecting the right and left scale points passes above the point for zero correction, the correction is zero. With temperatures below  $35^\circ$ , the connecting line cannot pass below the figure 0.9 on the inner scale because this figure represents a solution saturated with  $\text{AgIO}_3$  at  $35^\circ$ .

For general application to the iodometric chloride method, in which  $[\text{IO}_3^-]_0$  is measured gasometrically (1), colorimetrically (3), or by titration other than with 0.02303 N thiosulfate, the figures on the left and inner scales of the nomogram can be converted into terms of millimoles of dissolved iodate per liter by multiplying the values on both scales by  $100 \times 0.02303/6$ , or 0.3838.

In applying the reaction between  $\text{AgIO}_3$  and  $\text{Cl}^-$  to chloride determination one cannot determine a "reagent correction" by performing a blank analysis with water in place of the chloride solution, and measuring the dissolved  $\text{IO}_3^-$  as the correction. Under these conditions the solubility of the  $\text{AgIO}_3$  would not be depressed by  $\text{IO}_3^-$  formed by the reaction of  $\text{AgIO}_3$  and  $\text{Cl}^-$ , and the filtrate would contain an amount of  $\text{IO}_3^-$  which would correspond to the full  $\text{AgIO}_3$  solubility, and would therefore be greater than the true correction for the depressed  $\text{AgIO}_3$  solubility in analyses of chloride solutions. The reagents can be tested by analyses of standard chloride solutions, of which the one capable of most precise preparation appears to be standard hydrochloric acid made from constant boiling  $\text{HCl}$  by the method of Hulett and Bonner (5).

#### SUMMARY

In determining chloride by the measurement of the  $\text{IO}_3^-$  that goes into solution as a result of the reaction,  $\text{AgIO}_3 + \text{Cl}^- \rightleftharpoons \text{IO}_3^- + \text{AgCl}$ , correction for dissolved  $\text{AgIO}_3$  becomes significant ( $>0.5$  per cent) when  $\text{Cl}^-$  concentration falls below 6 mM at  $20^\circ$  or 10 mM at  $35^\circ$ . A nomogram is presented for calculating the correction from the temperature and the concentration of total dissolved  $\text{IO}_3^-$ .

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# A STUDY OF THE NORMAL DISTRIBUTION OF ASCORBIC ACID BETWEEN THE RED CELLS AND PLASMA OF HUMAN BLOOD

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A number of investigations (2, 3, 6, 7, 10) within the past 10 years has demonstrated the significance of determining ascorbic acid in the whole blood or white blood cells in assessing the vitamin C nutriture of individuals. These studies have shown (a) that the blood cells may contain ascorbic acid when there is no vitamin in the serum (2, 3), (b) that scurvy does not appear until the cellular ascorbic acid is zero (2, 3), and (c) that the cellular (2, 3) or whole blood (6, 7, 10) values are closely correlated with the total bodily ascorbic acid. Because of the continued widespread use of ascorbic acid determinations on serum or plasma in nutritional surveys, it seemed that a study undertaken to examine the interrelation of ascorbic acid levels in the serum (plasma), red cells, and whole blood would contribute to an understanding of the data already amassed. Such an investigation was further indicated by the lack of agreement among those who have already explored this interrelation as to whether the red blood cell or the serum contained the larger amount of ascorbic acid.

The present report deals with a study of the interrelations of the ascorbic acid concentrations in the plasma, red cells, and whole blood, as determined in the cases of twenty-six healthy adults.

## *Methods*

Throughout this study the ascorbic acid concentration of whole blood and of plasma was determined by the 2,4-dinitrophenylhydrazine method of Roe and Kuether (9). This method assays both ascorbic and dehydroascorbic acid.

The subjects used in the study were healthy men and women living on an ordinary diet. During the course of the investigation the diet of a few of the subjects was supplemented, for experimental purposes, with crystalline ascorbic acid. In all cases venous blood was obtained by venipuncture after 12 to 15 hours of fasting. The blood was immediately heparinized.

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Hematocrits were determined with Wintrobe tubes. The ascorbic acid concentration of the cells was calculated from the formula

$$A_c = (A_{wb} \times 1 - A_p V_p) / V_c$$

where  $A_c$ ,  $A_{wb}$ , and  $A_p$  represent the ascorbic acid concentration of the cells, whole blood, and plasma respectively, and  $V_p$  and  $V_c$  represent the plasma and cell volumes respectively.  $V_c$  includes the buffy layer and hence  $A_c$  refers to total (red + white) blood cell ascorbic acid content.

### Results

Table I summarizes the data collected on the eighteen healthy male and eight healthy female subjects. In all, thirty-eight determinations were made on these subjects. Essentially, the range of values we studied included the range of values reported by other investigators. The results will be presented in two parts.

(a) *Relation between  $A_c$  and  $A_p$* —Because ascorbic acid is a water-soluble substance, we felt that a truer picture of the distribution of ascorbic acid between cells and plasma would be obtained if the ascorbic acid concentrations were expressed in terms of mg. per 100 ml. of plasma and red blood cell water. In making the necessary calculations we used the data given by Bodansky (1) on the water content of human serum and red cells.

Accordingly the data in Table I were recalculated in terms of ascorbic acid per 100 ml. of  $H_2O$  and were charted. The straight line

$$A_p = A_c - 0.45 \quad (a)$$

where  $A_p$  and  $A_c$  are ascorbic acid concentrations, in mg. per 100 ml. of plasma and cell water respectively, was fitted by inspection. We felt that the application of the more refined statistical methods was unwarranted in view of the small population used in this study. This equation satisfactorily describes the trend of our data. From the equation it is seen that when the ascorbic acid concentration in the plasma and that in the cells are expressed in relation to water, the cells contain more ascorbic acid than the plasma under fasting (equilibrium) conditions over the entire range of ascorbic acid concentrations in plasma reported in this study.

When the ascorbic acid concentration is expressed in mg. per cent (as in Table I), the relation between  $A_c$  and  $A_p$  becomes

$$A'_p = 1.43 A'_c - 0.72 \quad (b)$$

Expressed in this way, the amount of ascorbic acid in the cells is greater than the amount in the plasma only when  $A_p$  is less than 1.55 mg. per cent. Above this level there is more ascorbic acid in the plasma (because of the greater amount of water in the plasma than in the cells).

TABLE I

*Ascorbic Acid Concentrations in Whole Blood, Plasma, and Red Blood Cells of  
Twenty-Six Healthy Subjects before Breakfast*

Subject	Hematocrit	Whole blood ascorbic acid	Plasma ascorbic acid	Cellular ascorbic acid	$A_p:A_c$
	<i>per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>	<i>mg. per cent</i>	
A. R.*	43.8	0.35	0.25	0.45	1.8
	47.1	0.45	0.30	0.55	1.8
*	44.0	0.50	0.30	0.70	2.3
	47.3	0.80	0.65	0.95	1.5
J. Pe.*	53.9	0.60	0.50	0.65	1.3
*	51.2	0.60	0.50	0.65	1.3
	54.3	0.70	0.55	0.85	1.5
	50.5	1.25	1.20	1.25	1.0
	51.6	1.90	2.00	1.80	0.9
J. Po.	47.7	0.60	0.30	0.90	3.0
H. G.	47.4	0.70	0.50	0.95	1.9
W. H.	47.0	0.70	0.60	0.80	1.3
L. Z.	51.6	0.80	0.70	0.90	1.3
F. C.	46.7	0.80	0.70	0.90	1.3
P. C.	46.8	0.80	0.65	0.95	1.5
G. N.	50.6	0.80	0.75	0.80	1.1
*	47.7	1.00	1.10	0.90	0.9
M. B.	44.2	0.85	1.00	0.65	0.65
D. D.	50.4	0.85	0.90	1.00	1.1
C. E.	50.0	0.90	0.75	1.05	1.4
L. H.	48.6	0.90	0.85	0.95	1.1
L. M.	45.8	0.95	0.95	0.95	1.0
M. S.	44.5	1.05	0.95	1.15	1.2
F. S.	44.4	1.10	1.10	1.10	1.0
	48.1	1.20	1.05	1.30	1.2
	45.6	1.50	1.30	1.80	1.4
F. B.	47.2	1.25	1.30	1.20	0.9
P. R.	43.4	1.25	1.20	1.35	1.1
	44.2	1.95	1.80	2.15	1.2
M. L.	43.9	1.40	1.35	1.45	1.1
R. J.	51.1	1.45	1.70	1.20	0.7
A. H.	41.9	1.55	1.45	1.60	1.1
	42.4	1.85	1.95	1.70	0.9
N. R.	47.4	1.65	1.70	1.60	0.95
P. K.	46.4	1.65	1.60	1.70	1.1
M. P.	43.4	1.70	1.85	1.50	0.8
M. M.	46.0	1.75	1.40	2.15	1.5
R. C.	43.4	1.85	1.90	1.80	0.95

\* Blood obtained after a breakfast of toast and coffee; fasting with respect to ascorbic acid.

(b) *Relation between  $A_w$  and  $A_c$ :*  $A_p$ —From equation (a) and the normal hematocrit we can calculate what the relation between  $A_w$  and  $A_c$ :  $A_p$

should be. Thus from  $A_p = A_c - 0.45$  and  $A_{wb} = 0.62 A_p + 0.38 A_c$  (normal hematocrit corrected for water content of whole blood, plasma, and cells (1)), we obtain

$$\frac{A_c}{A_p} = \frac{A_{wb} + 0.28}{A_{wb} - 0.17} \quad (c)$$

From our original data recalculated in terms of mg. per 100 ml. of  $H_2O$ , we plotted  $A_c:A_p$  versus  $A_{wb}$  and fitted a curve based on equation (c). This equation satisfactorily described the relationship. In other words, the ratio of the concentration of ascorbic acid in the blood cells to the concentration of ascorbic acid in the plasma is related to the concentration of ascorbic acid in the whole blood. Below  $A_{wb} = 1.5$  mg. per 100 ml. of  $H_2O$ , the dependency of the value of the ratio  $A_c:A_p$  upon  $A_{wb}$  is most significant, for in these ranges the greater concentration of ascorbic acid in the cells assumes a larger rôle in determining the value of this ratio than in the range of values where  $A_{wb}$  is greater than 1.5.

A shift in the ratio  $A_c:A_p$  with changing whole blood ascorbic acid content can be demonstrated when a single subject is followed at intervals before, during, and after saturation with ascorbic acid. Subject J. Pe. (Table I) was saturated with ascorbic acid, according to the criterion of Heineman (6), taking 1500 mg. of ascorbic acid in 24 hours. After saturation, the ratio decreased. During the following month, the ratio increased again as the whole blood ascorbic acid decreased. In another subject, who altered his diet so as to obtain a large amount of ascorbic acid from natural sources, we were able to demonstrate a decreased ratio (in a test about 1 month after the dietary alteration) as the whole blood ascorbic acid content increased (Subject A. R., Table I). Thus when one individual is followed through a period of changing ascorbic acid nutrition, we find the same relation between  $A_c:A_p$  and  $A_{wb}$  as when several individuals, each differing with respect to ascorbic acid nutriture, are compared.

We believe that the peculiar form of the relation between  $A_c:A_p$  arises from the facts that (1)  $V_p > V_c$  and that (2) when  $A_p = 0$ ,  $A_c = 0.45$ . Since the cells have a greater ascorbic acid content than does the plasma, as the plasma concentration approaches zero, the ratio  $A_c:A_p$  rapidly approaches infinity.

#### DISCUSSION

Our finding that the ratio of the concentration of ascorbic acid in the cells to that in the plasma depends upon the ascorbic acid concentration in whole blood confirms the findings of other workers who have studied this problem. In 1938 Heineman (6) reported that in fasting subjects

the ratio of ascorbic acid in the cells to that in the plasma was always greater than 1 and that this ratio increased as the whole blood ascorbic acid decreased. Butler and Cushman (2) state that the ratio  $A_c:A_p$  varies with the state of vitamin C nutrition. Recently Roe, Kuether, and Zimler (10) reported that, when the whole blood ascorbic acid was less than 0.6 mg. per cent, the whole blood contained more ascorbic acid than the plasma; when the whole blood ranged between 0.6 and 0.9 mg. per cent, the ascorbic acid content of whole blood and plasma was approximately equal, and when the whole blood exceeded 0.9 mg. per cent, the ascorbic acid concentration in whole blood was less than that of the plasma.

The fact that the distribution of ascorbic acid between the cells and the plasma is related to the whole blood level of ascorbic acid in part explains the disagreement in the literature concerning whether the ratio,  $A_c:A_p$ , is greater or less than 1. Examination of the reported data reveals that only a few of the investigators have studied the values of this ratio over the whole range of normally occurring ascorbic acid levels. This fact is clearly brought out by plotting on one chart all the available data from the literature. In general, the observed values follow closely the theoretical curve (equation (c)) describing the relation between  $A_c:A_p$  and  $A_{wb}$ . The relatively wide scatter which obtains in such a plot can be attributed reasonably to differences in methodology. Some workers (2, 8, 12) did not estimate the combined ascorbic and dehydroascorbic acid concentrations, whereas others (5, 7, 10, 11, 13) have estimated the total ascorbic acid content. Some investigators (2, 8, 12) have estimated the cellular ascorbic acid by direct analysis; others (5, 6) have calculated the cellular ascorbic acid from the hematocrit (see Butler and Cushman (2) for a critique of some of the methods used).

Clinical experience supports the present results, for it is now known that when the plasma contains no ascorbic acid (experimental scurvy) the cells contain ascorbic acid until the patient is scorbutic (2, 3). The white blood cells and platelets seem to cling even more avidly to the ascorbic acid than do the red cells (2).<sup>1</sup> Experiments on the nature of this cellular binding of ascorbic acid will be reported in a subsequent communication.<sup>2</sup> The results of these experiments suggest that ascorbic acid forms a relatively stable bond with a protein fraction of the red cell. In addition, studies which are not yet completed suggest that ascorbic acid forms a loose bond with a protein of the plasma.<sup>2</sup>

<sup>1</sup> In this connection Cuttle's (4) observation is of interest. This worker found that in leucocytosis the whole blood contained more ascorbic acid than did the plasma. As the white count returned to normal, the whole blood contained less ascorbic acid than the plasma.

<sup>2</sup> Sargent, F., and Forbes, W. H., to be published.

## SUMMARY

1. Thirty-eight determinations of ascorbic acid in the whole blood, plasma, and red blood cells were made on eighteen healthy males and eight healthy females.

2. When the ascorbic acid concentration is expressed in mg. per 100 ml. of blood cells or plasma, the relation between  $A_c$  and  $A_p$  is approximately  $A'_p = 1.43 A'_c - 0.72$ .

3. When the ascorbic acid concentration is expressed in mg. per 100 ml. of  $H_2O$ , the relation between  $A_c$  and  $A_p$  is approximately  $A_p = A_c - 0.45$ .

4. The ratio of the concentration of ascorbic acid in the cells to that in the plasma depends upon the concentration of ascorbic acid in the whole blood. This relation is approximately

$$A_c : A_p \quad \begin{array}{l} A_{wb} + 0.28 \\ A_{wb} - 0.17 \end{array}$$

where  $A$  is expressed in mg. per 100 ml. of  $H_2O$ .

5. We conclude that the disagreement in the literature concerning the distribution of ascorbic acid between cells and plasma (serum) is largely due to the fact that the various workers have not investigated the same range of whole blood ascorbic acid concentrations.

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## DEHYDROPEPTIDASE ACTIVITY IN CERTAIN ANIMAL AND PLANT TISSUES

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In 1932, Bergmann and Schleich (1) observed that glycerol extracts of swine kidney and pancreas possess the capacity of hydrolyzing glycyl-dehydrophenylalanine (glycyl- $\alpha$ -aminophenylacrylic acid) to glycine, ammonia, and phenylpyruvic acid. To the enzyme system responsible, whose distinction from dipeptidase, carboxypeptidase, and aminopeptidase was demonstrated, they gave the designation of dehydropeptidase. This enzyme system was not further investigated until recently, when, by using acetyldehydroalanine, chloroacetyldehydroalanine, and glycyldehydroalanine as substrates, it was noted that (1) glycyldehydroalanine was hydrolyzed with great rapidity in aqueous extracts of all animal and plant tissues studied, yielding products which included equivalent amounts of ammonia and pyruvic acid (2-4); (2) acetyldehydroalanine and chloroacetyldehydroalanine were hydrolyzed in extracts only of kidney and liver and of a few plant tissues to yield equivalent amounts of ammonia and pyruvic acid; (3) this distribution in different tissues capable of splitting glycyldehydroalanine and chloroacetyldehydroalanine suggested the existence of at least two different dehydropeptidases, for one of which, designated dehydropeptidase I, the former substrate was suitable, and for the other, designated dehydropeptidase II, the latter substrate was suitable (2). This distinction has since been established by Shack who separated the two enzymes by fractional alcohol precipitation at low temperature.<sup>1</sup>

Furthermore, it has been found that the aliphatic dehydropeptides possess a characteristic absorption in the ultraviolet region which disappears as the substrates are hydrolyzed; accordingly, an alternative method to that of the chemical determination of ammonia and pyruvic acid is available for following the course of dehydropeptidase activity in tissue preparations (2-4). Finally, the ready enzymatic degradation of cystine peptides (2) and of di(glycylamino)propionic acid (5, 6) to products which include equivalent amounts of ammonia and pyruvic acid suggests possible physiological precursors of the dehydropeptides.

The purpose of the present communication is to supplement these findings (1) by reporting the relative susceptibility of a wide variety of dehydropeptides, some of them new, to enzymatic hydrolysis by a number of animal

<sup>1</sup> Shack, J., personal communication.

and plant tissues, and by human serum; (2) by describing the characteristic absorption spectra of peptides of dehydrophenylalanine in the ultraviolet region, together with a method of following the enzymatic hydrolysis of these dehydropeptides based upon this characteristic absorption; and (3) by describing a new synthesis of N-chloroacylated peptides of dehydroalanine.<sup>2</sup>

### Procedure

#### *New Synthesis of Certain Dehydropeptides*

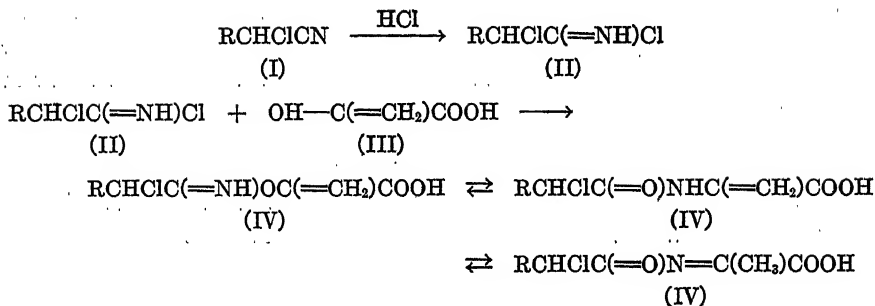
Bergmann and Grafe (8) described a method of preparing chloroacetyldehydroalanine in about a 20 per cent yield by heating a mixture of chloroacetamide and pyruvic acid *in vacuo* under a reflux condenser. Considerable difficulty has been encountered in this laboratory with this synthesis, only two successful preparations being achieved out of some two dozen attempts (9). A simple, reproducible, and nearly quantitative yield of chloroacetyldehydroalanine was achieved by mixing chloroacetonitrile with pyruvic acid in the presence of dry HCl gas (10). This method was subsequently successfully applied to the preparation of a new dehydropeptide, namely DL- $\alpha$ -chloropropionyldehydroalanine, through the use of  $\alpha$ -chloropropionitrile. On amination, the two halogenated dehydropeptides yielded the corresponding glycyldehydroalanine and DL-alanyldehydroalanine, respectively. This last compound is the first dehydropeptide prepared which possesses asymmetric carbon atoms in addition to free amino and carboxyl groups.<sup>3</sup>

The reaction between nitrile (I) and pyruvic acid in the presence of HCl gas may be due to the primary formation of the imino chloride (II) which, condensing with the enolic form of pyruvic acid (III) forms the corresponding dehydropeptide (IV).

The reaction appears at the present time to be limited to  $\alpha$ -chloronitriles, for acetonitrile or  $\beta$ -chloroacetonitrile mixed with pyruvic acid did not react.

<sup>2</sup> Recently, Fruton, Simmonds, and Smith (7) have investigated the effect of incubating cultures of *Escherichia coli* with various acetyldehydroamino acids, including acetyldehydrotyrosine, acetyldehydrophenylalanine, acetyldehydroleucine, and acetyldehydroalanine. The hydrolysis of these compounds was followed spectrophotometrically by noting the decrease in the absorption band in the ultraviolet region for each compound. Only acetyldehydrotyrosine and acetyldehydroalanine were appreciably decomposed during the growth of the organisms.

<sup>3</sup> The several dehydropeptides prepared by Doherty, Tietzman, and Bergmann, although containing asymmetric carbon atoms, are all acylated at the amino group (11).



*Chloroacetyldehydroalanine*—7.6 gm. of redistilled chloroacetonitrile were mixed with 10.6 gm. of redistilled pyruvic acid, chilled, and saturated with dry HCl gas. After standing for 1 hour at 5°, the mixture became a solid mass of crystals of nearly pure chloroacetyldehydroalanine. The mixture was allowed to stand in ice for another 12 hours, and was then washed on a suction filter with dry ether. The yield was 14 gm. or 86 per cent of the theory, based on the nitrile, m.p. 156°; N 8.3 per cent found, 8.6 per cent theory. A single crystallization from acetone raised the melting point to 162°, uncorrected; N 8.6 per cent found, 8.6 per cent theory. Bergmann and Grafe give 162–165°, corrected, as the melting point of their compound. The preparation, like that made according to the method of Bergmann and Grafe, gave in aqueous solution the characteristic ultraviolet absorption curve described by Carter and Greenstein (3).

*Glycyldehydroalanine*—The chloroacetyldehydroalanine so obtained was treated with aqueous ammonia in the usual manner (8) and an 80 per cent yield of long, glistening prisms of glycyldehydroalanine was obtained. M.p. 191°; N 19.2 per cent found, 19.4 per cent theory. The ultraviolet absorption spectrum was identical with that reported earlier (3).

*DL- $\alpha$ -Chloropropionyldehydroalanine*—9.0 gm. of redistilled  $\alpha$ -chloropropionitrile, prepared by the dehydration of  $\alpha$ -chloropropionylamide (12), were mixed with 10.6 gm. of redistilled pyruvic acid, chilled, and saturated with dry HCl gas. After standing in the ice chest overnight at 5°, a mass of crystals of the dehydropeptide had separated. The mixture was transferred to the filter with dry ether, washed several times, and dried. Yield, 12 gm. or 68 per cent of the theory; m.p. 130°, uncorrected. The compound readily crystallizes from acetone in flat prisms, m.p. 132°, uncorrected; N 7.6 per cent found, 7.8 per cent theory.

*DL-Alanyldehydroalanine*—10 gm. of the above compound were dissolved in 100 cc. of water saturated with ammonia at 5°. After standing 5 days at 25°, the solution was filtered and evaporated to a thick syrup *in vacuo*. The residue was taken up in a small amount of water and treated with a



large volume of absolute alcohol. Two repetitions of this procedure yielded small prismatic crystals of DL-alanyldehydroalanine, 4.5 gm., or 50 per cent of the theory. The compound begins to darken at 186°, but does not melt up to 230°.

Calculated, C 45.5, H 6.4, N 17.8; found, C 45.3, H 6.2, N 17.7

Both DL- $\alpha$ -chloropropionyldehydroalanine and DL-alanyldehydroalanine possess nearly identical absorption spectra in the ultraviolet region with

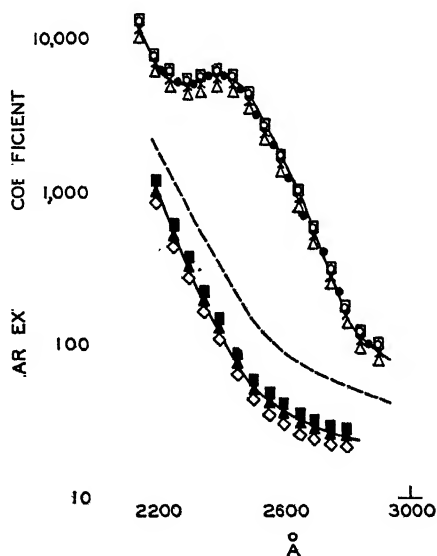
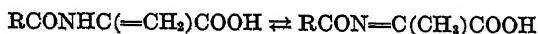


Fig. 1. Absorption spectra at pH 7.0 of aliphatic dehydropeptides (upper curve), pyruvic acid (middle, broken curve), and analogous saturated peptides (lower curve), all at  $1.7 \times 10^{-4}$  M.  $\times$  acetyldehydroalanine,  $\circ$  chloroacetyldehydroalanine,  $\bullet$  glycyldehydroalanine,  $\triangle$   $\alpha$ -chloropropionyldehydroalanine,  $\square$  alanyldehydroalanine,  $\blacktriangle$  acetylalanine,  $\blacksquare$  chloroacetylalanine,  $\diamond$  glycylalanine.

a maximum at 2400 A. Furthermore, the curves are nearly identical with those for acetyldehydroalanine, chloroacetyldehydroalanine, and glycyldehydroalanine (Fig. 1). It is evident that the shape of the curves is practically independent of the nature of the N-acyl radical, and this characteristic absorption may be attributed to the capacity of these compounds to exist in the following tautomeric equilibrium (3).



The difference in the amount and characteristics of the absorption between the dehydropeptides and those of their saturated analogues is noteworthy.

*Other Dehydropeptides and Peptides Studied*—Acetyldehydroalanine was prepared according to the method of Bergmann and Grafe (8) with the modifications reported by Gonçalves, Price, and Greenstein (4). N-Acetaminocinnamic acid (acetyldehydrophenylalanine) (13), chloroacetyldehydrophenylalanine (14), glycyldehydrophenylalanine (14), chloroacetyl-DL-alanine (15), glycyl-DL-alanine (15), chloroacetyl-DL-phenylalanine (16), and glycyl-DL-phenylalanine (16) were prepared according

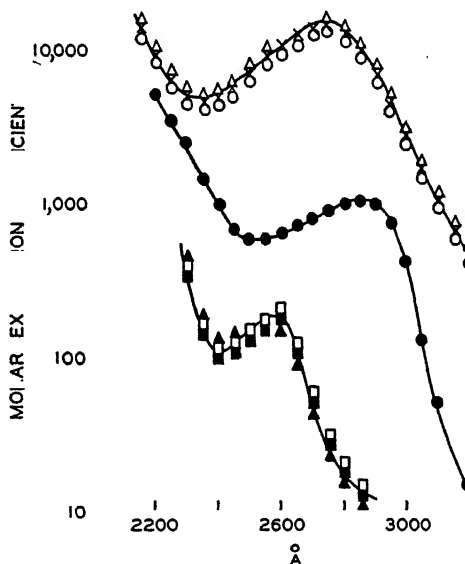


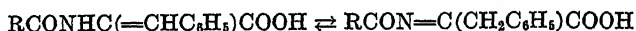
FIG. 2. Absorption spectra at pH 7.0 of aromatic dehydropeptides ( $5 \times 10^{-5}$  M, upper curve), phenylpyruvic acid ( $1.7 \times 10^{-3}$  M, middle curve), and analogous saturated peptides ( $1.7 \times 10^{-3}$  M, lower curve). X acetyldehydrophenylalanine,  $\Delta$  chloroacetyldehydrophenylalanine,  $\circ$  glycyldehydrophenylalanine,  $\square$  acetylphenylalanine,  $\blacktriangle$  chloroacetylphenylalanine,  $\blacksquare$  glycylphenylalanine.

to the references cited. Acetylalanine and acetylphenylalanine were prepared by the usual procedure of treating the amino acid with acetic anhydride (*cf.* (17)). All compounds were checked by their melting points and total nitrogen analysis.

#### *Ultraviolet Absorption Spectra of Dehydrophenylalanine Peptides*

As earlier reported (3) and as noted in Fig. 1, the peptides of dehydroalanine possess a characteristic absorption in the ultraviolet region, with a maximum at 2400 A. The peptides of dehydrophenylalanine also possess a characteristic absorption in the ultraviolet region, but the shape of these

curves is markedly different from that of the aliphatic dehydropeptides (Fig. 2). The saturated phenylalanine peptides possess nearly identical spectra with a maximum at about 2600 Å, which maximum may be attributable to the phenyl group. The unsaturated phenylalanine peptides possess a considerably greater absorption, with a maximum at 2750 Å, and the shape of the absorption curves, like that of the aliphatic dehydropeptides, is independent of the nature of the N-acyl radical. This characteristic absorption, too, may be attributed to the existence of the dehydropeptides in tautomeric forms.

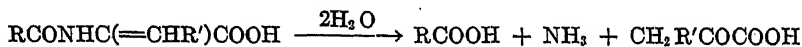


It is probable, however, that with this class of compounds the equilibrium lies far to the left, because of the tendency of the phenyl group to hold the side chain double bond in a position conjugated with the aromatic ring, thus preventing the tautomeric shift of the double bond to the carbon-nitrogen position.<sup>4</sup>

#### *Enzymatic Determinations*

Fresh aqueous extracts of various homogenized rat and plant tissues, as well as human serum, were the source of the enzymes studied. The digests consisted of 1 cc. of tissue extract at the desired concentration or 1 cc. of serum plus 2 cc. of 0.15 M sodium borate buffer at pH 8.1, plus 1 cc. of substrate solution at 0.025 M for all substrates except chloropropionyldehydroalanine and alanyldehydroalanine, which were at 0.05 M. Blanks on the extracts were determined by substitution of 1 cc. of water for the substrate solution, and the enzymatic activities noted were corrected for these blanks. The chloroacetyl peptide solutions were brought to pH 7.0 by addition of dilute NaOH. All substrates were completely stable in water over several weeks standing, as is revealed by their absorption curves in the ultraviolet region (Figs. 1 and 2).

The dehydropeptides are hydrolyzed under the influence of dehydropeptidases to products which include equivalent amounts of ammonia and keto acid (2, 4).



<sup>4</sup> The absorption spectra of acetyldehydrotyrosine and of acetyldehydrophenylalanine at pH 2 and 11 were recently described by Fruton, Simmonds, and Smith (7). The maximum extinction coefficients in acid were, respectively, 21,100 at 3050 Å and 15,400 at 2825 Å, and in alkali 23,050 at 3300 Å and 13,840 at 2725 Å. At 2575 Å, the maximum extinction coefficient of acetylphenylalanine was 250 at pH 2 and 240 at pH 11. From Fig. 2, at pH 7.0, the maximum extinction coefficient of acetyldehydrophenylalanine is 15,000 at 2750 Å, and of acetylphenylalanine 200 at 2600 Å.

The activity may be followed (1) chemically by measuring the rate at which ammonia and keto acid are produced, and (2) spectrophotometrically by measuring the rate at which the dehydropeptide bond is hydrolyzed through the progressive loss in the characteristic absorption of the substrate in the ultraviolet region (3, 4). Both methods yield identical and parallel results (4). The progressive loss in the characteristic absorption curve of acetyldehydroalanine in rat kidney digests was shown by Gon-

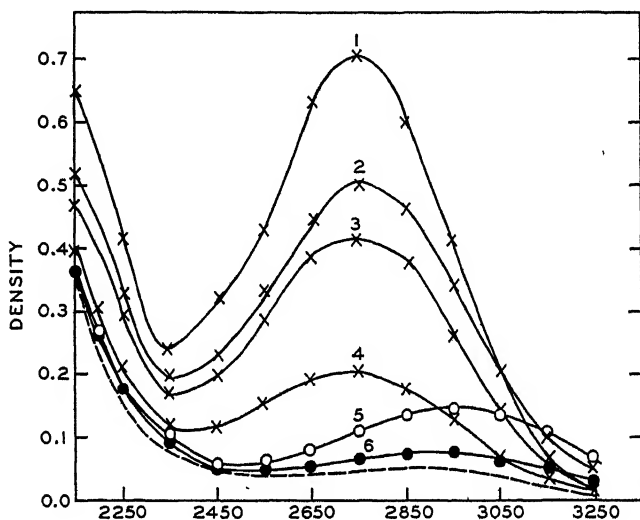


FIG. 3. Progressive change in absorption of glycyldehydrophenylalanine in rat kidney digests. The digests consisted of 1 cc. of extract equivalent to 50 mg. of fresh tissue, 2 cc. of borate buffer at pH 8.1, and 1 cc. of either water or 0.025 *M* substrate. Aliquots of digest diluted 1:125 with water prior to readings. Cells 1 cm. in length. Digestion periods, Curve 1, zero minutes, Curve 2, 5 minutes, Curve 3, 12 minutes, Curve 4, 30 minutes, Curve 5, 45 minutes, Curve 6, 62 minutes. The broken line refers to phenylpyruvic acid.

galves, Price, and Greenstein (4). In similar fashion, the progressive loss in the characteristic absorption curve of glycyldehydrophenylalanine in rat kidney digests is shown in Fig. 3. Curve 1 is that of the substrate; the bottom curve is that of phenylpyruvic acid which is the only product of the hydrolysis possessing a characteristic absorption of its own (maximum at 2850 A (4)). Toward the end of the digestion, the shape of the absorption curve approaches that of phenylpyruvic acid, just as in the case of the aliphatic dehydropeptides it approaches that of pyruvic acid (3).

Usually, only one wave-length is selected to follow the decomposition

of the dehydropeptides. For the aliphatic dehydropeptides the rate of fall in the absorption at 2500 Å was employed (3, 4). From the present results, the hydrolysis of the dehydrophenylalanine peptides is best followed spectrophotometrically by the rate of decrease of the 2750 Å band. Enzymatic activity is estimated as usual over the range in which this decrease is linear with time (3).

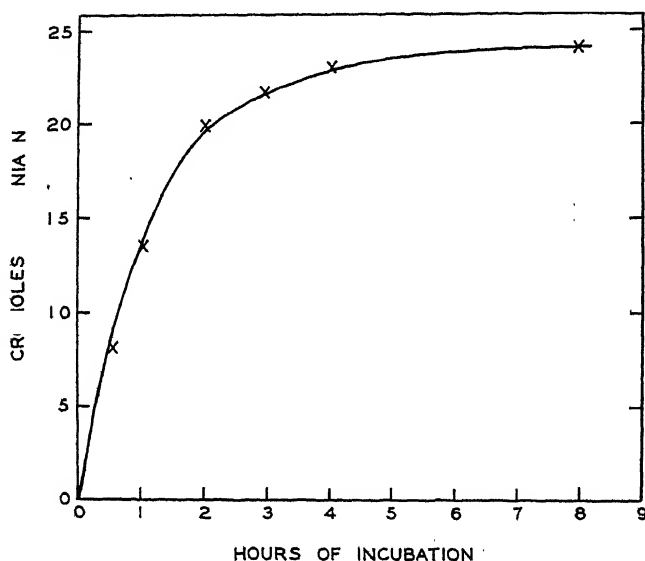


Fig. 4. Evolution of ammonia above controls in digests of DL-alanyldehydroalanine with aqueous extracts of Kentucky Wonder bean seeds. The digests consisted of 1 cc. of extract containing 0.7 mg. of total N, 2 cc. of borate buffer at pH 8.1, and 1 cc. of either water or 0.05 M substrate. Complete hydrolysis of both optical forms should yield 50 micromoles of ammonia N.

#### *Hydrolysis of DL-Alanyldehydroalanine*

In order to see whether both optical forms of DL-alanyldehydroalanine are enzymatically hydrolyzed in tissue preparations, this substrate was digested with aqueous extracts of rat kidney, rat liver, and bean seeds (Kentucky Wonder variety) under conditions which reveal maximum hydrolysis (Fig. 4). The evolved ammonia was determined by the aeration procedure described (2). The liver extracts contained 1 mg. of total N per cc., the kidney extracts, 0.25 mg. of total N per cc., and the digests were made up as described in Fig. 3. From a half hour period of digestion to 8 hours, the ammonia N evolved above the extract controls was a constant 23 to 24 micromoles. The results from the digests of this substrate with the animal and plant tissues studied reveal that only one of the two optical

isomers of DL-alanyldehydroalanine is enzymatically hydrolyzed. Until each optical form is synthesized and separately studied in the tissue digests, it cannot be said with certainty which one of the forms in the DL compound is hydrolyzed under these conditions, although it would be expected that the natural or L form would be the susceptible component. With the relatively dilute tissue extracts employed, little if any D- or L-amino acid oxidase activity is present, and the possible contribution to the evolved ammonia in the digests by oxidative desamination of the liberated alanine is negligible.

A similar study under maximum conditions of hydrolysis for DL- $\alpha$ -chloropropionyldehydroalanine was impracticable because of the very low rate of hydrolysis of this compound.

#### *Susceptibility of Various Dehydropeptides*

The relative rate of hydrolysis of available dehydropeptides by extracts of various animal and plant tissues was determined by noting the rate of evolution of ammonia in the digests by the method described (2). Suitable dilutions of the tissue extracts were made with water so as to yield within the 1st hour of digestion a nearly linear relation between hydrolysis and time. The activity of each tissue is then expressed in terms of micromoles  $\times 10$  of substrate hydrolyzed per hour per mg. of total nitrogen per cc. of extract used (3). Neither glycine nor glycylglycine yields ammonia under the present digestion conditions. Experiments with kidney digests with the various substrates under anaerobic conditions were conducted in the modified Thunberg tubes described earlier (18). The data are collected in Table I.

DL-Alanyldehydroalanine and glycyldehydroalanine are rapidly hydrolyzed by all of the tissues studied. Glycyldehydrophenylalanine is hydrolyzed at a considerably slower rate. Chloroacetyldehydroalanine and DL- $\alpha$ -chloropropionyldehydroalanine are hydrolyzed in extracts of kidney, liver, pancreas, mushroom, yeast, and sweet pea seeds. Acetyldehydroalanine is hydrolyzed in extracts of rat kidney and liver, as described earlier (4), and also to some extent in extracts of mushrooms. Neither acetyldehydrophenylalanine nor chloroacetyldehydrophenylalanine is hydrolyzed by extracts of any of the tissues studied. With kidney digests, the rate of hydrolysis of the substrates is apparently similar under both aerobic and anaerobic conditions (Table I).

#### *Dehydropeptidase Activity in Human Serum*

The sera of rats, rabbits, guinea pigs, and man are capable of hydrolyzing glycyldehydroalanine, and hence possess dehydropeptidase I activity (2). These experiments have since been repeated at a higher

pH, with a shorter period of digestion, and with the inclusion of the new dehydropeptide, DL-alanyldehydroalanine, as a substrate (Table II). Care was taken to secure blood without hemolysis, and the serum obtained by centrifuging the clot was free of hemoglobin. The samples were taken

TABLE I

*Susceptibility of Various Dehydropeptides in Rat and in Plant Tissue Extracts\**

Dehydropeptide	Micromoles $\times$ 10 substrate hydrolyzed per hr. per mg. total N per cc. extract in									
	Kidney†	Liver	Pancreas	Brain	Spleen	Muscle	Mushrooms	Yeast	Beans‡	Sweet pea seeds
DL-Alanyldehydroalanine	1350	52	722	220	412	58	750	35	360	401
Glycyldehydroalanine	1620	60	530	72	331	38	150	12	42	72
Glycyldehydrophenyl- alanine	520	12	132	0	23	0	8	0	0	0
DL- $\alpha$ -Chloropropionylde- hydroalanine	29	8	3	0	0	0	13	1	0	3
Chloroacetyldehydro- alanine	100	28	10	0	0	0	40	4	2	7
Chloroacetyldehydro- phenylalanine	0	0	0	0	0	0	0	0	0	0
Acetyldehydroalanine	18	3	0	0	0	0	6	0	0	0
Acetyldehydrophenyl- alanine	0	0	0	0	0	0	0	0	0	0

\* The digests consisted of 1 cc. of aqueous tissue extract plus 2 cc. of 0.15 M borate buffer at pH 8.1, plus 1 cc. of either water or 25 micromoles of substrate (50 micromoles of racemic substrates). Hydrolysis is measured in terms of ammonia nitrogen above the controls. Neither glycine nor glycylglycine yields ammonia under these conditions. Solutions of acetyl- and chloroacetyl peptides neutralized with NaOH before use. DL-Alanine yields no ammonia in digests with kidney under conditions used with DL-alanyldehydroalanine, since the extracts were very dilute and the periods of digestion very short.

† Identical digests with the various substrates under anaerobic conditions yielded practically identical results.

‡ Fleischmann brand.

§ Kentucky Wonder variety (seeds).

|| Rate calculated on basis of one optical component.

from healthy, young, normal subjects, two women and four men, and were allowed to stand in the ice chest to clot for 16 hours before use. Dehydropeptidase activity was measured by determining the ammonia evolved over the serum controls in digests of the substrates with the serum.

Under the conditions used, DL-alanyldehydroalanine was readily hydrolyzed by all of the samples of serum tested, to nearly 50 per cent of

the susceptible form of the dehydropeptide. The hydrolysis of glycyldehydroalanine and of chloroacetyldehydroalanine is nearly equal, and occurs to a much smaller extent than that of alanyldehydroalanine. The experiments described in Table II are obviously preliminary in nature and designed to form the basis of later, more extensive studies on the extent of the hydrolysis of the dehydropeptides, particularly that of alanyldehydroalanine, in the serum of patients in various disease states.

TABLE II  
*Dehydropeptidase Activity in Human Serum\**

Subject	Ammonia nitrogen evolved from		
	DL-Alanyldehydroalanine	Glycyldehydroalanine	Chloroacetyldehydroalanine
	micromoles	micromoles	micromoles
M. L. E. ....	11.1	2.0	1.9
V. P. ....	10.8	2.0	2.0
C. D. ....	14.3	2.2	1.9
M. B. ....	10.5	2.0	1.9
W. D. ....	8.6	1.9	1.8
F. M. L. ....	10.8	2.1	2.0

\* The digests consisted of 1 cc. of serum plus 2 cc. of 0.15 M borate buffer at pH 8.1, plus 1 cc. of either water or 25 micromoles of substrate (50 micromoles of DL-alanyldehydroalanine). Solutions of chloroacetyldehydroalanine neutralized with NaOH before use. Incubation period 2 hours at 37°. No ammonia evolved under these conditions from digests of serum with glycine, glycyglycine, or DL-alanine.

#### DISCUSSION

Dehydropeptidase I is one of the most ubiquitous and most active of intracellular enzymes. It is present in all animal and plant tissues studied (2-4) (Table I) and is particularly active in tumors (3). About 24 mg. of glycyldehydroalanine are hydrolyzed per hour at pH 8 in aqueous extracts of rat kidney equivalent to 100 mg. of fresh tissue (*cf.* Table I). It appears probable from the activity ratios in various tissues that DL-alanyldehydroalanine may not be a substrate for dehydropeptidase I, but rather for some closely related enzyme. In the four plant tissues studied, DL-alanyldehydroalanine is hydrolyzed at a considerably faster rate than is glycyldehydroalanine (Table I). For both substrates the descending order of hydrolysis rate in the various tissues is as follows: (1) rat tissues, kidney, pancreas, spleen, brain, liver, and muscle; (2) plant tissues, mushrooms, sweet pea seeds, bean seeds, and yeast. It is possible that glycyldehydrophenylalanine is also hydrolyzed by dehydropeptidase I, but at a considerably slower rate than is either of the aliphatic dehydropeptides



mentioned. The relative order of hydrolysis rate of glycyldehydrophenylalanine in the various tissues studied is similar to that of the aliphatic peptides (Table I). The presence of the  $\beta$ -phenyl group thus lowers the enzymatic susceptibility of the dehydropeptide.

Dehydropeptidase II activity is generally lower than that of dehydropeptidase I. The rate at which chloroacetyldehydroalanine is hydrolyzed in kidney extracts is about 14 per cent, acetyldehydroalanine about 1 per cent, of that of glycyldehydroalanine (Table I). It seems probable that DL- $\alpha$ -chloropropionyldehydroalanine is hydrolyzed by dehydropeptidase II, or some closely related enzyme, but at a slower rate than is chloroacetyldehydroalanine. The relative effectiveness of the various tissues in hydrolyzing the chloro-substituted dehydropeptides is, in descending order, as follows: (1) rat tissues, kidney, liver, and pancreas; (2) plant tissues, mushrooms, sweet pea seeds, yeast, and bean seeds. Spleen, muscle, and brain of several animal species are lacking in or low in their capacity to hydrolyze chloroacetyldehydroalanine (2) (Table I), and this, together with the fact that the relative order of effectiveness of the various tissues is different for glycyldehydroalanine and for chloroacetyldehydroalanine, is among the reasons for the belief in the presence of at least two dehydropeptidases (2). Acetyldehydroalanine is a substrate for dehydropeptidase II, and is appreciably hydrolyzed only in extracts of kidney, liver, and mushrooms. The relatively high activity of both dehydropeptidases I and II in mushrooms is noteworthy.

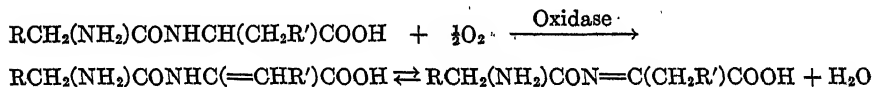
Neither acetyldehydrophenylalanine nor chloroacetyldehydrophenylalanine is hydrolyzed in preparations of the tissues studied. Perhaps, just as the presence of the  $\beta$ -phenyl group in glycyldehydrophenylalanine diminishes its susceptibility to the action of dehydropeptidase I, so too might the presence of this group in acetyldehydrophenylalanine and in chloroacetyldehydrophenylalanine reduce the susceptibility of these compounds to the action of dehydropeptidase II.

The presence of the  $\beta$ -phenyl group, however, in DL-phenylalanine or in glycyl-DL-phenylalanine does not diminish the action of D-amino acid oxidase on these substrates below that on corresponding substrates containing DL-alanine (19).

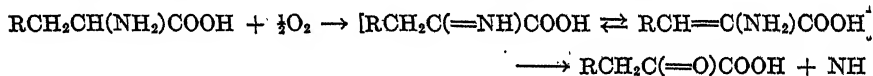
The rôle of the dehydropeptidases in intracellular protein metabolism is not yet clearly defined, largely because the mode of origin of the natural substrates for these enzymes is not definitely known. The ready susceptibility of favorably constituted peptides with an  $\alpha,\beta$  double bond to enzymatic hydrolysis in a wide variety of tissues suggests, as Bergmann *et al.* first pointed out (14), that the oxidative metabolism of amino acids may begin while they are still bound in peptide linkage. Further evidence has been advanced in support of this view by studies on the relative desamina-

tion of isomeric dipeptides and tripeptides of DL-alanine and of DL-leucine (19). With kidney homogenates under aerobic conditions, glycyl-DL-leucine and glycyl-DL-alanine yield ammonia, whereas DL-leucylglycine and DL-alanylglycine do not. Of the isomeric tripeptides of DL-leucine and glycine, namely DL-leucylglycylglycine, glycyl-DL-leucylglycine, and glycylglycyl-DL-leucine, only the last yields ammonia when incubated with oxygenated kidney homogenates. Thus, only when the susceptible amino acid in the peptide chain is in a position where its  $\alpha$ -carboxyl group is free will it yield ammonia, presumably after oxidative dehydrogenation of the  $\alpha, \beta$ -hydrogen atoms. Bergmann and Schleich (1) had shown that, whereas glycylglycyldehydrophenylalanine was hydrolyzed by dehydropeptidase, glycyldehydrophenylalanylglycine was not affected.

The reaction whereby dehydropeptides may arise by an oxidative  $\alpha, \beta$  dehydrogenation of the saturated peptide is



This reaction, involving the amino acid moiety of the peptide, is analogous to that believed to occur in the oxidative desamination of the free amino acids, which according to Knoop (20), and later Dakin (21) and Bergmann and Stern (22), involves the formation of the  $\alpha$ -imino acid which exists in tautomeric equilibrium with the  $\alpha$ -aminoacrylic acid derivative.



The compounds enclosed in brackets are unstable and have never been isolated. When, however, such compounds are N-acylated, they are entirely stable in water, and are frequently designated dehydropeptides.

Another mechanism whereby dehydropeptides may arise under physiological conditions is by the enzymatic desulfuration of cystine while it is in peptide linkage, yielding thereby the corresponding dehydropeptide, hydrogen sulfide, and sulfur (2).

A third mechanism whereby dehydropeptides may arise *in vivo* is based upon the ready *in vitro* condensation of amides and pyruvic acid to form the corresponding dehydropeptides (8) (*cf.* (23)). It was noted by Greenstein and Carter (24) that the desamidation of glutamine and asparagine was considerably increased in liver digests to which pyruvic acid was added. Since the reaction occurred at a pH range at which glutaminase activity was negligible, and since the pyruvic acid could be nearly quantitatively recovered at the end of the experiment (*cf.* (25)), it was suggested that the desamidation of glutamine and of asparagine in the presence of pyruvic

acid involves two steps, namely (1) the condensation of the amide with the keto acid to form a dehydropeptide, followed by (2) the hydrolysis of the latter by dehydropeptidase to yield the corresponding amino acid, ammonia, and the regenerated keto acid.

A fourth proposed mechanism is based upon the possibility that not 1 mole but 2 moles of amide may condense with pyruvic acid to yield the corresponding di(acylamino)propionic acid. Bergmann and Grafe (8), Nicolet (26), and Shemin and Herbst (27) had prepared compounds of this type, and noted that, on treatment with warm glacial acetic acid, 1 molecule of amide is liberated from the di(acylamino)propionic acid, leaving the dehydropeptide. On the basis of these observations, Gonçalves and Greenstein (5, 6) prepared  $\alpha,\alpha$ -di(glycylamino)propionic acid and found that it was enzymatically hydrolyzed by extracts of rat kidney to yield a maximum of 1 mole of ammonia and 1 mole of pyruvic acid per mole of substrate. These results were interpreted in part as due to an enzymatic decomposition of the substrate to glycynamide and glycyldehydroalanine, followed by the hydrolysis of the latter compound (5).

Further knowledge bearing on the possible modes of origin of the dehydropeptides, and of the enzymes which hydrolyze these unsaturated substrates, will depend in part on their isolation, purification, and characterization.

#### SUMMARY

A method has been described for synthesizing chloroacetyldehydroalanine and the new peptide, DL- $\alpha$ -chloropropionyldehydroalanine, by condensing the respective nitriles with pyruvic acid in the presence of dry HCl gas. From these, the corresponding glycyldehydroalanine and DL-alanyldehydroalanine were obtained by treatment with aqueous ammonia.

The ultraviolet absorption spectra are described for the new dehydropeptides, and for the dehydropeptides containing a  $\beta$ -phenyl group adjacent to the double bond, and comparisons drawn with the spectra for the analogous saturated peptides. A spectrophotometric method for following dehydropeptidase activity when glycyldehydrophenylalanine is used as substrate is described.

The relative susceptibility to enzymatic hydrolysis by aqueous extracts of a wide variety of rat and of plant tissues was studied of the following substrates: acetyldehydroalanine, chloroacetyldehydroalanine, glycyldehydroalanine, DL- $\alpha$ -chloropropionyldehydroalanine, DL-alanyldehydroalanine, acetyldehydrophenylalanine, chloroacetyldehydrophenylalanine, and glycyldehydrophenylalanine. Only one of the two optical isomers of DL-alanyldehydroalanine is hydrolyzed. The rate of hydrolysis of the peptides with a free  $\alpha$ -amino group, is, in each tissue, generally higher than that of

peptides lacking this group, while the presence of a  $\beta$ -phenyl group in the molecule of the substrate exerts in general a retarding effect on the dehydropeptidase.

Human serum readily hydrolyzes DL-alanyldehydroalanine and, to a lesser degree, glycyldehydroalanine and chloroacetyldehydroalanine.

Possible modes of biological origin of the substrates for dehydropeptidases are described.

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# THE IN VITRO METABOLISM OF $\Delta^4$ -ANDROSTENEDIONE-3,17 TO TESTOSTERONE, *cis*-TESTOSTERONE, AND SEVERAL UNIDENTIFIED STEROIDS\*

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It has been demonstrated in this laboratory (1) that  $\Delta^4$ -androstenedione-3,17, *cis*-testosterone, and small amount of other unidentified steroids are formed when testosterone is incubated with liver slices. In order to elucidate further the course of the metabolism of these steroids, 2000 mg. of  $\Delta^4$ -androstenedione-3,17, the chief metabolite of testosterone (1), has been incubated in lots of 100 mg. per 10 gm. of liver slices with sterile precautions. The procedure, gross extraction, and fractionation were identical to those employed in the first study.

## Results

The ketonic fraction (Table I) weighed 1855 mg., indicating that about 17 per cent<sup>1</sup> of the added  $\Delta^4$ -androstenedione-3,17 had been converted to either non-ketonic steroids or unrecoverable compounds. Bioassay of this fraction by the rat method of Kochakian (2) showed a 129 per cent increase in androgenic activity, indicating that some of the  $\Delta^4$ -androstenedione-3,17 had been metabolized to a biologically more active ketosteroid or ketosteroids. This material was separated into non-hydroxy and hydroxy ketones by treatment with succinic anhydride. The non-hydroxy ketonic fraction yielded 935 mg. of pure  $\Delta^4$ -androstenedione-3,17, or only 47 per cent of the added substrate (Table I). No other steroids could be isolated from the residues, although etiocholanedione-3,17 or etioallocholanedione-3,17 (androstenedione-3,17) or both might be expected to be present if the double bond of Ring A was reduced.

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Presented before the Division of Biological Chemistry of the American Chemical Society at New York, September, 1944.

<sup>1</sup> The crude ketonic material contains about 10 per cent of non-steroid impurities (*cf.* (1)).

The half succinates of the hydroxy ketonic fraction weighed 861 mg., which is equivalent to 624 mg. of monohydroxy ketones. These substances were hydrolyzed first by potassium bicarbonate and finally by potassium hydroxide (3). The milder hydrolysis yielded mainly testosterone and the stronger hydrolysis both testosterone and *cis*-testosterone. The amount of testosterone isolated was quite large, 230 mg., and provides an explanation for the increase in androgenic activity noted in the total ketonic fraction. The amount of *cis*-testosterone was small, 5.5 mg. of pure and 11.4

TABLE I

*Materials Isolated after Incubation of 2000 Mg. of  $\Delta^4$ -Androstenedione-3,17 with Rabbit Liver Slices*

	Weight	M.p.
	mg.	°C.
Fat-soluble	14,083	
Acetone-soluble	7,946	
Non-ketonic	5,908	
Cholesterol	715	147-148
17( $\alpha$ )-Hydroxy steroid	3	215-216.5
Total ketonic	1,855	
Non-hydroxy ketonic	1,130	
$\Delta^4$ -Androstenedione-3,17	935	169-170
Hydroxy ketonic	624*	
Testosterone	230	151-153
<i>cis</i> -Testosterone	5.5†	220-221
17-Ketohydroxy steroid, Compound A	3	82-85; 143-149
" " " A-2	10.2	48-53; 124-142
" " " A-3	(5.0)	(Crude)
" " " B	1	194.5-195.5
" " " B-2	2	198-203
17( $\alpha$ )-Hydroxy ketosteroid, Compound C	2.5	206-211
17( $\alpha$ )-Hydroxy ketosteroid, Compound C-2	1.5	199-209

\* Calculated from the succinate as monohydroxy steroid.

† 11.4 mg. of crude substance.

mg. of crude product, but was adequate for definite characterization. In addition to these two compounds smaller amounts of three other ketosteroids were isolated, but only in sufficient amounts to provide a partial characterization by means of melting point and color reactions. They were 17-ketosteroids but not identical to any of the known compounds. It is to be noted that the amount of material isolated after the saponification of the succinates represents only 40 per cent of the amount calculated to be present.

The non-ketonic fraction yielded a large amount, 715 mg., of cholesterol and also a very small amount, 3 mg., of a compound which melted at 215–216.5° and possessed a 17( $\alpha$ )-hydroxyl group as indicated by color tests (4).

The aqueous material remaining after the extraction of the original extracts with benzene and ether was extracted with ethyl acetate; the impure material, 158 mg., showed no androgenic activity in the castrated rat (2), but gave a positive glucuronic acid test with Tollens' reagent, a positive Zimmermann reaction (5), a negative Pincus reaction (6), and a positive color test for the  $\Delta^5$ -3-hydroxyl group (7), which suggest the presence of a compound resembling dehydroisoandrosterone glucuronide. No crystalline substance could be isolated.

In order to be sure that there was complete removal of androgens from the phospholipide fraction, a bioassay on the rat was performed. The results were negative.

The above isolation procedures were repeated on a micro scale with the use of only 80 mg. of  $\Delta^4$ -androstenedione-3,17 for the incubation. The chief metabolites, testosterone and *cis*-testosterone, and  $\Delta^4$ -androstenedione-3,17 were recovered in approximately the same proportions as with the use of the larger amounts of substrate.

#### DISCUSSION

Contrary to expectation,  $\Delta^4$ -androstenedione-3,17 apparently is not an intermediate in the conversion of testosterone by the rabbit liver to less active androgens, *e.g.* androsterone. Indeed, the reverse seems to be the route of choice;  $\Delta^4$ -androstenedione-3,17 is converted more readily to a highly active androgen, testosterone, than to any other identifiable steroid.

The isolation of *cis*-testosterone as well as testosterone in the same amount formed and recovered after the incubation of testosterone with rabbit liver slices (1) indicates that during the reduction of the 17-keto group, both 17( $\alpha$ )- and 17( $\beta$ )-hydroxyl compounds are formed, but the former in much greater amounts than the latter. Furthermore, the testosterone "recovered" in the previous study contained some testosterone newly formed from the reduction of its metabolite,  $\Delta^4$ -androstenedione-3,17.

The isolation of only compounds which were formed by the alteration of the 17-keto group suggests that this position in  $\Delta^4$ -androstenedione-3,17 as well as in testosterone (1) is most susceptible to the oxidation-reduction enzymes of the rabbit liver. It must be borne in mind, however, that considerable material was unaccounted for, about 20 per cent, which may be indicative of highly degraded material. Furthermore, variations in procedure, *e.g.* amounts of substrate, time of incubation, pH, etc., may alter the equilibrium and possibly reveal other metabolites.

The rapidity with which rabbit liver metabolizes androgens, only 2.5 hours, and the multiplicity of compounds formed strongly suggest that the



multiple reactions (*cf.* (8, 9)) obtained by the administration of a steroid hormone are dependent upon the number and nature of the metabolites formed by the liver and other tissues. Thus, any derangement in liver function would greatly alter the response elicited by a given steroid.

#### EXPERIMENTAL<sup>2-5</sup>

*Recovery of  $\Delta^4$ -Androstenedione-3,17 from Non-Hydroxy Ketonic Fraction*—The non-hydroxy ketonic fraction, 1130 mg., was crystallized from ether-acetone and pentane to give 763 mg. of  $\Delta^4$ -androstenedione-3,17, m.p. 168–169°. A mixed melting point with authentic  $\Delta^4$ -androstenedione-3,17 showed no depression.

*Analysis*— $C_{19}H_{26}O_2$ . Calculated, C 79.68, H 9.15; found, C 79.70, H 9.09

The dioxime melted at 141–143° and showed no depression when mixed with an authentic sample of  $\Delta^4$ -androstenedione-3,17 dioxime, m.p. 142–143°.

A thorough search of the remaining non-hydroxy fraction for other ketonic steroids was made by chromatographic separation of the combined mother liquor residue on Brockmann's aluminum oxide. An additional 167 mg. of  $\Delta^4$ -androstenedione-3,17 were isolated, but no other compounds could be found. The total amount of  $\Delta^4$ -androstenedione-3,17 recovered was 935 mg.

#### *Fractional Hydrolysis of Succinates and Chromatographic Separation of Hydroxy Ketonic Steroids*

*Potassium Bicarbonate Hydrolysis*—The hydroxy ketonic succinates, 861 mg., on hydrolysis with potassium bicarbonate (3) yielded a yellow oil, 224 mg., which was chromatographed by the magnesium silicate-Celite procedure (1).<sup>3</sup>

Fractions 1 to 10, 18.5 mg., gave negative color tests and resisted attempts at crystallization.

\* The steroids used in this study were provided by Ciba Pharmaceutical Products, Inc.

<sup>3</sup> Equal parts by weight of magnesium silicate and Celite No. 503. We are indebted to Dr. K. Dobriner for acquainting us with this adsorbent.

<sup>4</sup> All of the melting points were determined with Anschütz thermometers on an electrically heated aluminum block (unpublished) placed under a microscope. Calibration of the apparatus with standard substances indicated that true values were obtained as used.

<sup>5</sup> All analytical samples were dried over phosphorus pentoxide *in vacuo* at 110°. The carbon and hydrogen determinations were made by the micromethod by Dr. D. Ketcham, Research Laboratories, Eastman Kodak Company.

Eluates 12, 13, and 14, 127.3 mg., gave positive 17-hydroxyl tests, with the strong red fluorescence characteristic of testosterone. These three fractions, therefore, were pooled and recrystallized from methanol-water to give 87 mg. of crystals, m.p. 150–152°; a mixed melting point with testosterone showed no depression.

Eluate 22, 7.0 mg., gave a positive Zimmermann test and negative 17-hydroxyl and 17( $\beta$ )-hydroxyl tests. Recrystallization from dilute methanol yielded about 1 mg. of clean platelets which gradually changed to needles at 135–140°, giving a "pincushion" appearance; the needles melted sharply at 194.5–195.5°. The amount isolated was too small for characterization (Compound B).

Eluate 11, 7.7 mg., Eluates 15 to 21, 82.8 mg., and Eluate 23, 7.2 mg., gave a violet or brown-violet color in the Zimmermann test (5), a positive 17-hydroxyl test, and a negative 17( $\beta$ )-hydroxyl test (4), which indicated the presence of some testosterone. Attempts at crystallization failed. These fractions, therefore, were pooled, dissolved in 12 ml. of benzene, diluted with 6 ml. of pentane, and adsorbed on a 12  $\times$  35 mm. column of aluminum oxide (Brockmann). The column was eluted with benzene, benzene containing increasing amounts of methanol, and finally methanol. The first two eluates, 8.7 mg., gave negative color tests and could not be crystallized; they were discarded. Eluate 3, 13.1 mg., gave color reactions typical of a 17-ketosteroid (5) and similar to that of dehydroisoandrosterone (7). On recrystallization from methanol, 3 mg. of crystals (Compound A, Table I) were obtained which melted at 82–85°, resolidified, and remelted at 143–149°. Mixed melting points with dehydroisoandrosterone and also etiocholanol-3( $\alpha$ )-one-17, however, showed sharp depressions.

Eluates 4 to 15, 46.7 mg., yielded on fractional crystallization a total of 28 mg. of crystalline material, m.p. 142–152°; a mixed melting point with testosterone showed no depressions.

Eluates 16 and 17, 6.1 mg., as well as Eluates 18, 19, and 20, 122.1 mg., were treated separately because of the very strong violet-colored Zimmermann reaction. The negative Pincus test (6) indicated the absence of androsterone or its isomers. Crystalline material could not be obtained.

Eluate 21, 7.8 mg., an oil in which crystals slowly developed, showed a positive 17-hydroxyl test and a negative 17( $\beta$ )-hydroxyl test. The oil was removed by washing with acetone and the crystals, 2.5 mg., were recrystallized from ether in which they were only slightly soluble. The substance, Compound C, apparently a 17( $\alpha$ )-hydroxy ketone, melted at 206–211°.

The remaining eluates, Nos. 22 to 30, 19.1 mg., could not be crystallized.

A total of 115 mg. of testosterone and trace amounts of Compounds A, B, and C were isolated from the 224 mg. of hydroxy ketones, indicating a considerable loss of material.

*Potassium Hydroxide Saponification of Succinates of Hydroxy Ketonic Fraction*—The succinates, 495 mg., which had not been hydrolyzed with potassium bicarbonate were hydrolyzed with 5 per cent potassium hydroxide (3) to yield 234 mg. of a yellow resin-like material. The aqueous fraction containing the unhydrolyzed succinates and impurities, upon acidification, extraction, and evaporation yielded 76 mg. of a yellow oil. The hydroxy ketones, 234 mg., were dissolved in benzene and adsorbed on 10 gm. of magnesium silicate-Celite in a column  $24 \times 11$  mm. and eluted (1).

Eluates 1 through 6, 11.6 mg., could not be crystallized and gave negative color tests.

Elate 7, 5.0 mg., gave a violet Zimmermann reaction and responded in the Dirscherl and Zilliken test (7) and the Pincus test (6) as dehydroisoandrosterone, but crystalline material could not be obtained. In the color responses it resembled Compound A described previously (Compound A-3).

Eluates 8, 9, and 10, 108.1 mg., were apparently a mixture of testosterone, *cis*-testosterone, and an unknown 17-ketosteroid and were pooled for read-sorption (see below).

Fractions 11 through 13, 64.5 mg., were pooled and recrystallized from dilute methanol, giving 50 mg. of white needles melting at  $151^\circ$ . A mixed melting point with testosterone showed no depression.

*Analysis*— $C_{18}H_{28}O_2$ . Calculated, C 79.17, H 9.72; found, C 79.21, H 9.52

The benzoate melted at  $185$ – $190^\circ$  and on mixture with testosterone benzoate (m.p.  $192$ – $193^\circ$ ) melted at  $190.5$ – $192^\circ$ .

Fractions 14 through 19, 61.4 mg., and Fractions 21 through 23, 8.8 mg., apparently mixtures, were pooled for rechromatographing on aluminum oxide.

Fraction 20, 5.5 mg., yielded 2 mg. of platelets when recrystallized from acetone, which changed to needles at  $145$ – $150^\circ$  and melted at  $198$ – $203^\circ$ . This compound, No. B-2, seemed to be identical with Compound B.

*Chromatographic Separation of Fractions 8, 9, and 10*—Fractions 8, 9, and 10 were pooled and chromatographed (10).

Elate 1, 0.9 mg., was discarded. Eluates 2 and 3, 10.2 mg., gave an impure product when crystallization procedures were used. It sintered at  $48$ – $53^\circ$  and finally melted at  $124$ – $142^\circ$ . In the color tests used it appeared to be identical with Compound A (Table I). It could not be further characterized (Compound A-2).

From Eluates 4 through 10, 76.3 mg., were obtained 63 mg. of crystalline material, m.p.  $147$ – $150^\circ$ , which did not depress the melting point of testosterone.

Eluates 11 to 18, 11.4 mg., which on the basis of the color tests were

expected to yield *cis*-testosterone, gave 5.5 mg. of crystals from dilute methanol, m.p. 219–221°. The acetate melted at 109–111° and showed no depression when mixed with an authentic sample of *cis*-testosterone acetate.

*Analysis*— $C_{21}H_{30}O_2$ . Calculated, C 76.12, H 9.1; found, C 75.97, H 8.85

Eluates 19 and 20, 1.8 mg., failed to yield crystalline material.

*Chromatographic Separation of Fractions 14 to 19 and 21 and 22*—The residues, 78 mg., from Eluates 14 to 19 and 21 and 22 were adsorbed on aluminum oxide in the manner described above (10). An additional 2 mg. of testosterone and 1.5 mg. of a strongly adsorbed compound melting at 199–209° were obtained. The latter, Compound C-2, appeared to be identical with Compound C previously described.

### *Non-Ketonic Fraction*

*Saponification*—The non-ketonic fraction, 6318 mg., on hydrolysis with methanolic potassium hydroxide yielded 1185 mg. of neutral and 4720 mg. of acidic materials. The latter was not investigated.

*Chromatographic Analysis of Neutral Fraction*—The neutral material, 1185 mg., was subjected to chromatographic analysis with the magnesium silicate-Celite procedure.

A total of 715 mg. of cholesterol was isolated from Eluates 8 to 17. It melted at 147–148° and did not depress the melting point of an authentic sample of cholesterol.

Eluates 25 and 26, 24.4 mg., gave a positive test for the 17-hydroxyl group and a blue-green Pincus reaction. Since crystalline material could not be obtained by various procedures, it was subjected to chromatographic separation on aluminum oxide. One of the fractions yielded a few mg. of a crystalline substance which melted at 180–192°. It was recrystallized several times from dilute acetone to yield 3 mg. of a pure product which melted at 215–216.5°. The crystalline material gave a strong positive 17-hydroxyl test and a negative 17( $\beta$ )-hydroxyl test, indicating the presence of the 17( $\alpha$ )-hydroxyl group. A mixed melting point with androstanediol-3( $\alpha$ ),17( $\alpha$ ) (m.p. 220–222°) showed a 50° depression. Androstanediol-3( $\beta$ ),17( $\alpha$ ), m.p. 164–168°, and  $\Delta^5$ -androstenediol-3( $\beta$ ),17( $\alpha$ ), m.p. 179.8–181.4°, melt below that of the isolated compound. The remaining possible 17-hydroxy steroids (etioholanediol-3( $\alpha$ ),17( $\alpha$ ), isolated by Butenandt *et al.* (11) from urine) and etioholanediol-3( $\beta$ ),17( $\alpha$ ) were not available for comparison.

The other eluates did not yield crystalline material and gave negative tests for the 17-hydroxyl group, but several fractions eluted after cholesterol gave weak blue to blue-green colors in the Pincus test.

## SUMMARY

Incubation of a suspension of 2 gm. of  $\Delta^4$ -androstenedione-3,17 in a serum-buffer mixture with surviving rabbit liver slices for 2.5 hours at 37° resulted in the conversion of approximately half of the substrate to hydroxy ketonic steroids and a 129 per cent increase in androgenic activity (rat test). The major products isolated from the hydroxy ketonic fraction were 230 mg. of testosterone and 5.5 mg. (11.4 mg. of crude) of *cis*-testosterone. Three other hydroxy ketonic compounds were isolated in small amounts and partially identified. A small amount of a 17( $\alpha$ )-hydroxyl compound was isolated from the non-ketonic fraction. Approximately 17 per cent of the  $\Delta^4$ -androstenedione-3,17 could not be accounted for, and considerable material was lost in the many manipulative procedures necessary to isolate the pure compounds.

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# A COLORIMETRIC METHOD FOR THE DETERMINATION OF THE PROTEOLYTIC ACTIVITY OF DUODENAL JUICE

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The coupling of diazotized aryl amines with proteins in alkaline solution yield chromophoric protein derivatives. Digestion of a solution of such azoproteins with proteolytic enzymes results in the formation of colored components soluble in trichloroacetic acid. The intensity of the color in the trichloroacetic acid filtrate of the digested substrate is a function of the proteolytic activity of the enzyme solution and serves as the basis for the method here described.

## EXPERIMENTAL

*Preparation of Sulfanilamide-Azocasein*—Solution A, 50 gm. of reprecipitated, fat-free casein are dissolved with stirring in 1 liter of water containing 10 gm. of  $\text{NaHCO}_3$ . Solution B, 5.0 gm. of sulfanilamide are dissolved in 200 ml. of  $\text{H}_2\text{O}$  containing 6.0 ml. of 5.0 N NaOH. 2.20 gm. of  $\text{NaNO}_2$  are added. To the stirred solution 18.0 ml. of 5 N HCl are added and stirring is continued for 2 minutes. 18.0 ml. of 5 N NaOH are then added; the solution is stirred and added at once, with stirring, to Solution A.

The azoprotein is precipitated by acidification to pH 4.5, washed with water and alcohol, and air-dried.

The azocasein is a red-orange compound with an absorption maximum at 440  $\text{m}\mu$  (Fig. 1).

*Substrate Solution*—A stock solution of the substrate containing 25 mg. of azocasein and 5 mg. of sodium bicarbonate per ml. is prepared by dissolving 2.50 gm. of azoprotein in 50 ml. of 1.0 per cent  $\text{NaHCO}_3$  at 60° with stirring. The pH is adjusted to 8.3 and the solution diluted to 100 ml. with distilled  $\text{H}_2\text{O}$ . The stock solution is stored at 0°.

*Duodenal Juice*—The specimens are centrifuged at about 1500 R.P.M. for 10 minutes and the sample taken from the relatively homogeneous middle layer. 1 ml. of sample is diluted to 100 ml. with bicarbonate buffer (5 mg. per ml.), pH 8.3.

## Procedure

Each determination is set up in duplicate. The flask containing the substrate solution and a rack with the proper number of tubes are placed in a

water bath set at 38°. After the substrate solution has reached bath temperature, 1 ml. is pipetted into each of the tubes. 1 ml. of the diluted duodenal juice is then added to the tubes containing the substrate. A substrate blank is prepared by substituting 1 ml. of bicarbonate buffer for the test sample. At the end of 30 minutes the digestion is stopped and undigested azoprotein precipitated from the solution by the addition of 8 ml. of 5 per cent trichloroacetic acid to each tube, including the substrate blank tubes. The contents of each tube are filtered through paper. To a 5 ml. aliquot of the filtrate are added 5 ml. of approximately 0.5 N NaOH and the color is read on an Evelyn photoelectric colorimeter with the No. 440 filter. No fading of the color has been observed for at least 2 hours.

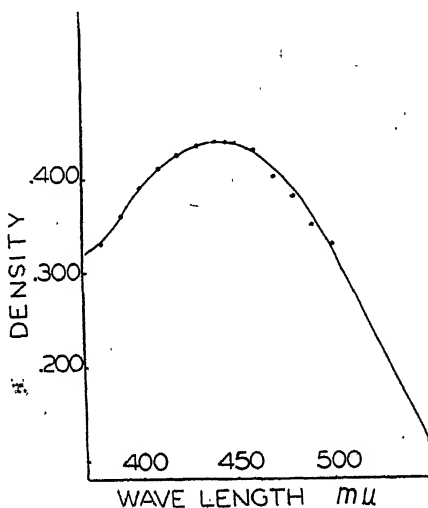


FIG. 1. Absorption curve of sulfanilamide-azocasein (0.25 mg. per ml.)

It has not been found necessary to run a blank on the enzyme solution; after the total dilution of 1:2000 and treatment with trichloroacetic acid, even the darkest samples have always shown 100 per cent light transmission.

#### Calculations

The enzymatic hydrolysis of a protein is a first order reaction, the velocity constant of which is expressed by the equation

$$K = \frac{1}{t} 2.3 \log \frac{C_1}{C_2}$$

where  $C_1$  and  $C_2$  are initial and final protein concentrations respectively after  $t$  minutes of digestion.

Since the color-concentration relationship of sulfanilamide-azocasein and its digestion products obeys Beer's law, optical density values may be substituted for  $C$  values. The initial concentration,  $C_1$ , is determined by adding 5 ml. of 0.5 N NaOH to a 5 ml. aliquot of a 1:200 dilution of the substrate and then reading the color of the final solution. The  $C_2$  value is determined by subtracting the optical density of the trichloroacetic acid filtrate from the  $C_1$  value. This is permissible, since a solution of azocasein which is completely digested (no trichloroacetic acid precipitate) has the same color intensity as an undigested sample.

TABLE I  
Sample Calculation of  $K$  Values

Tube No.		Per cent transmission	Optical density	Corrected for blank ( $d$ )	$C_2$ (5.920 - $d$ )	$\text{Log } \frac{C_1}{C_2}$	$K \times 10^{-7}$
1	Substrate blank	92	0.0362				
2	" "	92	0.0362				
3	Duodenal Juice 1	58 <sup>3</sup>	0.2328	0.1966	5.723	0.0147	11.3
4	" "	57 <sup>3</sup>	0.2384	0.2022	5.718	0.0151	11.6
5	Duodenal Juice 2	71 <sup>3</sup>	0.1442	0.1080	5.812	0.0080	6.1
6	" "	73	0.1367	0.1005	5.819	0.0079	6.1
7	Undigested substrate*	50 <sup>3</sup>	0.295	(5.900)*			
8	" "	50 <sup>2</sup>	0.297	(5.940)			
				(5.920 Average)			

\* The undigested substrate must be diluted 20 times more than the filtrate samples to be readable in the colorimeter; therefore, the optical density must be multiplied by 20 to yield the  $C_1$  value.

The velocity constant is calculated for the diluted solution and multiplied by the dilution factor to obtain the reaction constant for the undiluted juice. A sample of the calculations appears in Table I.

In these calculations the blank value has been subtracted from the optical densities of the filtrates only to show the magnitude of the blanks. In practice, the instrument is set to 100 per cent transmission with the blank. With a direct reading instrument, such as the Klett, the calculations are simpler still.

### Results

If the enzymatic digestion of the azocasein conforms to the equation for a monomolecular reaction, then the velocity constant rather than some arbitrary unit may be used as a measure of enzyme activity. From the kinetic equation it may be seen that  $\log (C_1/C_2)$  is directly proportional to  $t$ .



That this relation is followed under the conditions of the determination is shown in Fig. 2. With two dilutions of duodenal juice, a rectilinear relationship was found between  $\log (C_1/C_2)$  and time,  $t$ , in minutes.

The velocity constant of an enzyme reaction is a function of enzyme concentration. The data plotted in Fig. 3 reveal that under the conditions of the assay enzyme activity ( $\log (C_1/C_2)$ ) is directly proportional to the

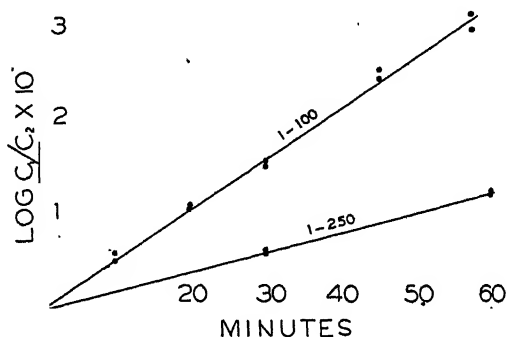


FIG. 2. Relationship of  $\log (C_1/C_2)$  to time,  $t$

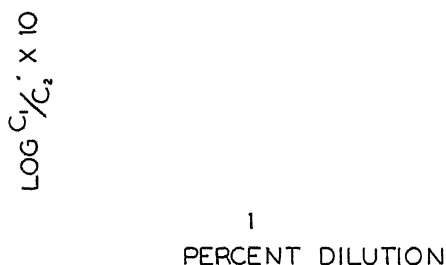


FIG. 3. Relationship of enzyme activity,  $\log (C_1/C_2)$ , to dilution of duodenal juice.

concentration in the duodenal juice. In the 1:50 dilution sample of Fig. 3, 10 per cent digestion had occurred. In other experiments the direct proportionality has been shown to exist in from 0 to 25 per cent digestion of the substrate, thereby permitting determinations to be made over a large range of dilutions. The direct relationship is especially satisfactory in that it permits recalculation to original juice strength merely by multiplication by the dilution factor.

## DISCUSSION

The method described has proved readily adaptable to the routine testing of clinical samples. It is simple, rapid, and sensitive and conforms to the theoretical monomolecular reaction equation. It has shown a high order of reproducibility; from the fourteen determinations run to yield the data in Fig. 2, in which digestion time and enzyme concentration were varied, an average velocity constant of 0.1198 was calculated, with an average deviation of  $\pm 0.0031$  or  $\pm 2.5$  per cent. A definite advantage of this method over many in which suspensions of insoluble substrates are employed is that the digestion is not influenced by particle size. Since the color of azocasein follows Beer's law, optical densities may be substituted for protein concentration, thus eliminating the necessity for the construction of nomographs. The color is stable and chromogenic reagents are not required for its development. Slight variations in substrate concentration of the stock solution are of minor significance, since in each determination the substrate concentration is determined and considered in the calculations.

The technique, in which a chromophoric protein substrate is employed, may be extended to the determination of other proteolytic enzymes. Sulfanilamide-azocasein is insoluble at acid pH and therefore unsuitable for peptic analysis. By the proper choice of aryl amine and protein, substrates may be prepared which are suitable for proteolytic enzymes in general. Further investigations are now in progress.

## SUMMARY

A colorimetric method for the determination of tryptic activity has been presented in which a soluble chromophoric protein substrate, sulfanilamide-azocasein, is employed.

The increase in color in a trichloroacetic acid filtrate of the digestion mixture is a function of enzyme activity.

The enzymatic hydrolysis satisfies the conditions for a monomolecular reaction and permits activity to be expressed in terms of the velocity constant.



# INHIBITORY ACTION OF VITAMIN P COMPOUNDS ON HYALURONIDASE

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In 1936 Szent-Györgyi and coworkers (1) described the isolation of a material which was capable of increasing capillary resistance in man, and which they called citrin or vitamin P. Subsequent work has shown that many substances have this property, and vitamin P activity has been attributed variously to flavones, flavanones, and flavonols.

Higby (2) found that crude hesperidin possessed vitamin P activity, but that upon purification it became inactive. He attributed the activity of the crude preparations to the presence of hesperidin in soluble form, as the chalcone. Majovski *et al.* (3) postulated that pure hesperidin was inactive because of its relative insolubility. Scarborough (4), however, found that hesperidin, as well as its aglycone hesperetin, was active in pure form. Rutin has been reported to increase capillary resistance in man (5), as have esculin and its aglycone esculetin (6).

The mechanism by which these and the other known vitamin P substances act to increase capillary resistance is unknown, although Lavollay (7) has postulated that they function as inhibitors of adrenalin oxidation, and that adrenalin itself or its first oxidation product is the true vitamin P.

It has been suggested that hyaluronidase plays a rôle in the maintenance of capillary strength. Duran-Reynals (8) reported that the permeability of the vascular system could be increased by preparations rich in hyaluronidase. More recently hyaluronidase has been considered as a factor "in accentuating capillary fragility rather than in inducing direct changes in capillary permeability" (9). The possibility is thus raised that the effect of vitamin P substances in increasing capillary resistance is due to an inhibition of the action of hyaluronidase. It appeared that an investigation of this point might bear fruitful results.

## EXPERIMENTAL

Hyaluronidase was prepared from bull testes by the method of Kass and Seastone (10). The hyaluronic acid used was prepared from bovine vitreous humor by the method of Seastone (11). The estimation of hyaluronidase activity was performed turbidimetrically (11).

For each assay two tubes were set up. Into one were pipetted 1.5 cc. of a solution of hyaluronic acid in 0.1 M acetate buffer, pH 6.0, plus 0.5 cc.

of a water solution of the substance whose effect was being tested. The other tube contained 2.0 cc. of a solution of the hyaluronidase in buffer. The two tubes were allowed to stand for 5 minutes at 37°, and their contents were then mixed; the mixture was incubated for  $\frac{1}{2}$  hour at 37°. At the end of this time 1 cc. of the contents of the tube was pipetted into a mixture of 3 cc. of 0.5 M acetate buffer, pH 4.2, and 1 cc. of acidified horse serum (11). Turbidity was allowed to develop for  $\frac{1}{2}$  hour and was then determined in the turbidimeter. The readings were converted to concentration of hyaluronic acid by reference to a standard curve. The concentration of hyaluronic acid in the reacting tubes was 0.2 mg. per cc.; the concen-

TABLE I  
*Inhibitory Action of Vitamin P Compounds on Hyaluronidase*

Compound	Per cent inhibition		
	0.1 mg. per cc.	1 mg. per cc.	0.1 mg. per cc. + ascorbic acid, 0.1 mg. per cc.
Ascorbic acid.....	25		
Dicoumarol.....	35		
Hesperidin .....	0	0	0
" (purified).....	0	0	40
" methyl chalcone.....	0	0	75
Rutin.....	0	70	65
Esculin.....	0	0	40
Esculetin.....	0	0	50
4-Methyl esculetin.....	0	0	55
Sulfanilamide.....	0	0	
Salicylic acid.....	0	0	0
Salicyluric acid.....		0	

tration of hyaluronidase was adjusted so as to bring the final reading to the flattest part of the standard curve.

Controls were run with and without hyaluronidase and with and without the substance whose effect was being tested. Results were obtained in a typical experiment by a comparison of four tubes, whose contents were as follows: (1) hyaluronic acid, (2) hyaluronic acid + hyaluronidase, (3) hyaluronic acid + hyaluronidase + test substance, (4) hyaluronic acid + test substance.

When combinations of substances were tested, additional controls were run.

The group of vitamin P substances tested included rutin, hesperidin, a sample of hesperidin purified by treatment with formamide, hesperidin methyl chalcone, esculin, esculetin, and 4-methyl esculetin. Other sub-

stances tested were ascorbic acid, salicylic acid, salicyluric acid, sulfanilamide, and dicoumarol. These compounds were tested at concentrations of 0.1 and 1.0 mg. per cc. Several of the vitamin P compounds, notably hesperidin and esculetin, were not completely soluble at the higher concentration and precipitated during the incubation, settling to the bottom of the tubes. In these cases the clear supernatant was pipetted into the diluted serum.

### *Results*

Of the vitamin P substances tested, only rutin showed an inhibitory action on hyaluronidase, and this only at high concentrations. The only compounds possessing activity at the lower concentration were ascorbic acid and dicoumarol. However, a combination of ascorbic acid and the vitamin P compounds showed a marked potentiation of inhibitory action, especially in the case of hesperidin methyl chalcone. The results are presented in Table I.

There was no synergistic effect noted when hesperidin methyl chalcone was tested in combination with dicoumarol.

### DISCUSSION

Of the vitamin P substances tested, only rutin showed any effect on hyaluronidase, and that only at a relatively high concentration. It is problematical whether this reaction can be considered to be specific; it may well be due to some impurity present in the rutin.

In the presence of ascorbic acid, however, the compounds having vitamin P activity manifest a well marked inhibitory action on hyaluronidase. It appears to be possible that a hyaluronidase-ascorbic acid combination is susceptible to inhibition by these compounds, while hyaluronidase itself is not. The inhibitory action of ascorbic acid is not entirely unexpected; this compound has long been known as a specific remedy against hemorrhagic lesions (12). It seems possible that its action is due to an inhibition of hyaluronidase, and that it is two-fold: first, a direct inhibition of hyaluronidase, and second a potentiation of the action of vitamin P. Because they seem to be closely associated in natural products, it is probable that a deficiency in ascorbic acid, one of whose symptoms is a marked increase in capillary fragility, will also be accompanied by a deficiency in vitamin P; there may thus be two factors to be considered here.

The fact that crude hesperidin showed no activity in the presence of ascorbic acid, while the sample of purified hesperidin did, is believed to be due merely to a concentration of the hesperidin in purification. The crude hesperidin contained a very considerable quantity of impurities, as is evidenced by the losses in the purification process; the purified hesperidin

thus contained more actual hesperidin per unit weight than did the crude form.

Heparin is known to be a specific inhibitor of hyaluronidase (13). In view of this it becomes of interest that dicoumarol, which also functions as an anticoagulant in the body, manifests this property. It is a question for speculation whether the anticoagulating properties of these compounds are related to their effect on hyaluronidase. It should be noted that heparin is a much more specific inhibitor than dicoumarol, or, indeed, than any of the compounds tested; it has been found to give greater inhibition at very much lower concentrations (14). Dicoumarol showed no synergism when it was tested with hesperidin methyl chalcone, indicating that this property is peculiar to ascorbic acid.

Salicylate has been reported to inhibit the spreading effect of India ink in rabbits injected with hyaluronidase (15). No inhibition was noted with this substance *in vitro*. Further, salicylic acid, a metabolite of salicylic acid, was found to be without effect. No synergism was noted between salicylic acid and ascorbic acid. This suggests that this synergism is limited to compounds having vitamin P activity, although, of course, extensive further work is necessary to prove this point.

#### SUMMARY

Ascorbic acid and dicoumarol were found to inhibit the action of hyaluronidase at concentrations of 0.1 mg. per cc. Of the other compounds tested, only rutin showed an inhibitory action, at a concentration of 1.0 mg. per cc.

Combining ascorbic acid with the vitamin P substances tested caused a marked potentiation of the inhibitory action. This was not true of a combination of dicoumarol and hesperidin methyl chalcone.

Neither salicylic acid nor salicylic acid had an inhibitory effect. There was no synergism between salicylic acid and ascorbic acid.

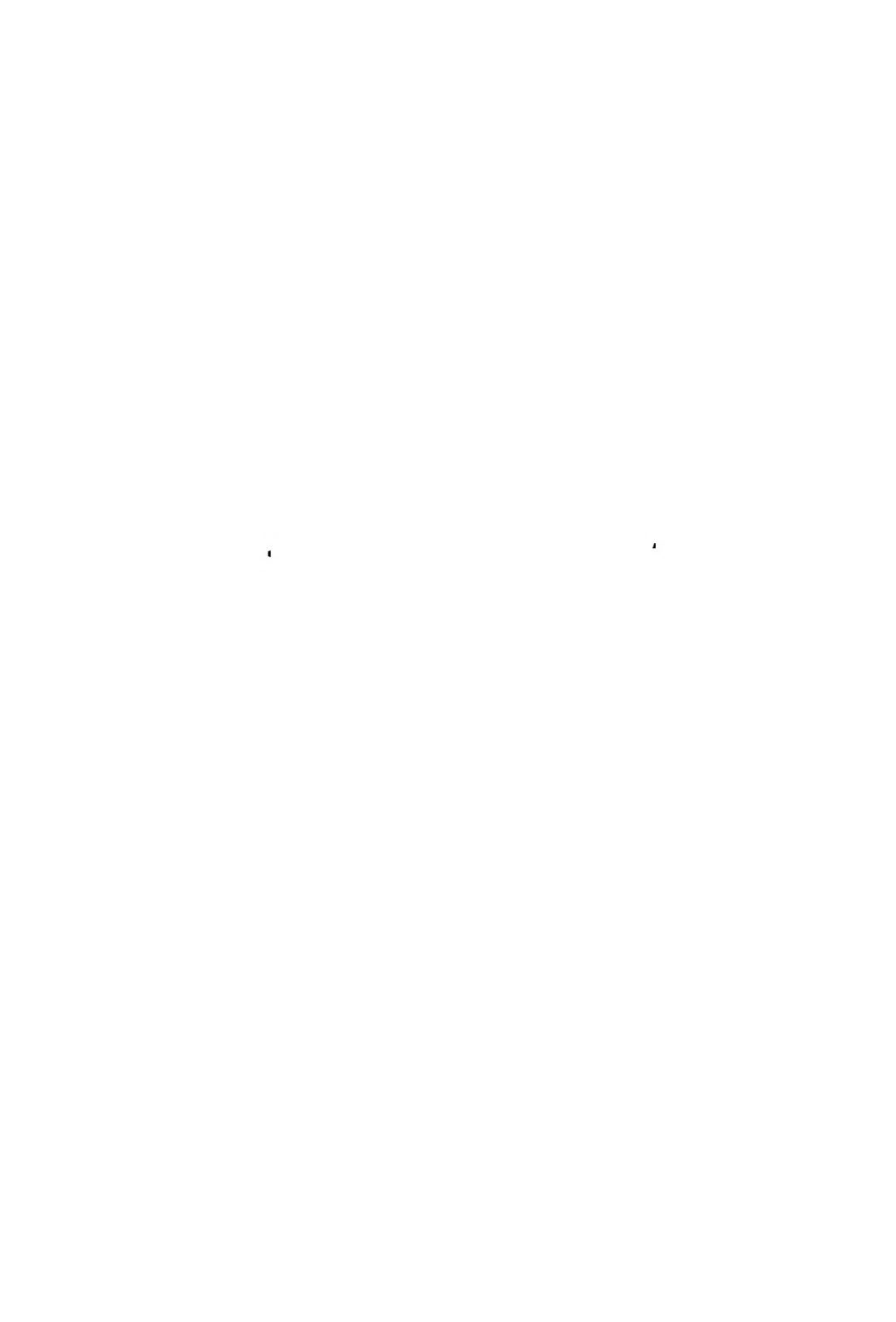
Sulfanilamide was without effect.

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# THE EFFECT OF THYROID ON THE CONVERSION OF CAROTENE INTO VITAMIN A\*

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That the thyroid gland might be involved in the metabolism of carotene was first suggested by von Noorden in 1907 when he observed a carotinemia to be associated with metabolic disturbances (1). Wendt (2) noted that patients with Graves' disease had very low serum vitamin A levels, even though their intake of carotene was adequate for normal individuals. Wohl and Feldman (3) observed that hypothyroid patients often showed a poor dark adaptation, and concluded that the thyroid was in some way concerned with carotene metabolism. Escamilla (4) and Mandelbaum *et al.* (5) observed a carotinemia associated with myxedema, and both conditions tended to clear up under treatment with thyroid substance.

Laboratory studies have likewise pointed toward the conclusion that a functioning thyroid gland is necessary for the animal to convert carotene into vitamin A. Kunde (6) observed xerophthalmia in thyroidectomized rabbits that had been fed an adequate diet containing enough carotene to satisfy the vitamin A requirement of a normal rabbit. Fasold and Heide-mann (7) showed that the vitamin A content decreased and the carotene content increased in milk from thyroidectomized goats. Recently Drill and Truant reported that carotene failed to prevent xerophthalmia in thyroidectomized rats, although preformed vitamin A furnished protection against these lesions (8). Experiments by Abelin (9), however, suggest that thyroxine may affect the metabolism of both carotene and vitamin A. Guinea pigs rendered hyperthyroid by the administration of thyroxine did not metabolize carotene or store vitamin A as well as did normal animals.

In the present study rats were depleted of vitamin A and at the same time brought into either the hypo- or hyperthyroid state. Standard amounts of carotene or vitamin A were then fed, and the storage of vitamin A in the livers and kidneys determined chemically.

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*Procedure*

Groups of weanling Sprague-Dawley rats were fed variations of the following diet low in vitamin A: casein 18, yeast 8, dextrin 65, salt 4 (10), cottonseed oil 5. The control group received only this low vitamin A diet. The other groups received additional supplements of thiourea, thiouracil, or desiccated thyroid. A hypothyroid condition was produced either by feeding thiourea mixed in the diet at a level of 0.5 per cent or by adding 2-thiouracil to the drinking water at a concentration of 0.1 per cent (11). A hyperthyroid state was produced by feeding 1 mg. of desiccated thyroid tissue per gm. of body weight per day (12). The dried tissue was suspended in water and fed by dropper. This supplement was prepared fresh every 3rd day and stored in a refrigerator. Attempts were also made to neutralize the effects of the thiourea and the thiouracil by the subcutaneous injection of 5 mg. of DL-thyroxine in dilute  $\text{NaHCO}_3$  daily. This amount permits approximately normal metabolic activity in young thyroidectomized rats (13, 14). The solution injected had a pH of 8.5 and contained 20 mg. of thyroxine per ml.

During the first weeks of the experiment, measurements of the oxygen consumption were made in a modification of the apparatus described by Schwabe and Griffith (15). Unfortunately the apparatus available was designed for mice and could no longer be used after the rats exceeded 70 gm. in weight. However, the dosages of supplements employed were sufficient to maintain states of hypo- or hyperthyroidism for many weeks, according to the data of others (16, 17). The present experiments lasted for about 6 weeks, 4 for the development of avitaminosis A and 2 more during which carotene or vitamin A was administered. The supplements of the thio compounds or of thyroid or thyroxine were continued throughout the depletion period and the period of vitamin supplementation.

When the rats ceased to grow and showed incipient ophthalmia, two to four animals from each group were killed for the analysis of vitamin A in the livers and kidneys. In no case was any of the vitamin detected. Half of each group was then given  $\beta$ -carotene, while the other half received vitamin A. The  $\beta$ -carotene was purified chromatographically and was dissolved in cottonseed (Wesson) oil at concentrations that furnished the desired daily dose in 3 drops of oil, which also contained 0.5 mg. of added  $\alpha$ -tocopherol. The vitamin A supplement consisted of the unsaponifiable fraction of halibut liver oil dissolved in cottonseed oil so that 3 drops contained the desired daily dose of vitamin A plus 0.5 mg. of added  $\alpha$ -tocopherol.

In a preliminary series the dosages were 20 i.u. of vitamin A or 60  $\gamma$  of  $\beta$ -carotene per rat daily. These amounts of vitamin and provitamin

resulted in total average stores of 21  $\gamma$  of vitamin A in the liver and kidneys of the control rats receiving the halibut oil concentrate and of 67  $\gamma$  in those receiving the  $\beta$ -carotene. In subsequent series the dosages were changed so that the control groups fed the provitamin or the vitamin would develop approximately equivalent stores of vitamin A in the liver and kidneys. The doses fed were 40  $\gamma$  of  $\beta$ -carotene or 40 i.u. of vitamin A per rat daily. The supplements were fed daily for 15 days and the animals were decapitated 24 hours after the last supplement was given. Livers and kidneys were removed and the organs analyzed colorimetrically for vitamin A, as outlined previously (18, 19).

### Results

The administration of thyroid substance increased oxygen consumption about 77 per cent above that observed in the control animals (Table I),

TABLE I  
*O<sub>2</sub> Consumption of Rats in Various States of Thyroid Activity*

Diet	O <sub>2</sub> consumption, liters per 24 hrs. per kilo body weight			Average
Low vitamin A	50.2	51.1	43.2*	48.2
“ “ “ + 1 mg. desiccated thyroid per gm. body weight per day	90.4	71.3	94.8	85.5
Low vitamin A with 0.5 % thiourea	33.5	32.5	41.5	35.8
“ “ “ 0.5 “ “ + 5 $\gamma$ DL-thyroxine per day	58.1	59.1		58.6
Low vitamin A with 0.1 % thiouracil in drinking water	35.4	38.8	38.2	37.5
As above + 5 $\gamma$ DL-thyroxine per day	51.8	58.1	57.8	55.9

\* Each figure represents three to five measurements taken on an individual animal.

whereas the feeding of thiourea or thiouracil resulted in average decreases in oxygen consumption of 26 and 22 per cent respectively. When thyroxine was administered along with the thio compounds, this decrease was counteracted and the consumption of oxygen was 16 to 21 per cent above that of control rats not receiving either type of agent.

The conditions producing these changes in basal rate also resulted in markedly different growth rates of the rats during both the depletion and supplementation periods (Table II). Rats receiving thiourea gained an average of only 30 gm. during the depletion period. Those receiving thiouracil grew approximately as well as the control animals on the low vitamin A diet, the gains being 43 and 44 gm. respectively. The differences in growth observed in the presence of thiouracil and thiourea are

probably due to the fact that a more nearly toxic dose of thiourea was used (12). The animals receiving thyroid grew at only a slightly slower rate than the controls, whereas the addition of thyroxine to the diet of rats fed thiourea increased growth somewhat, although the inhibition due to the thiourea was not completely overcome (Table II).

TABLE II  
*Storage of Vitamin A in Tissues of Hypo- or Hyperthyroid Rats  
Fed Carotene or Vitamin A*

Diet	Daily supplement	No. of animals	Average depletion period weight gain	Average supplement period weight gain	Mean liver vitamin A	Mean kidney vitamin A	Total vitamin A
			gm.	gm.	$\gamma$	$\gamma$	$\gamma$
Low vitamin A	60 $\gamma$ $\beta$ -carotene*	3	81	22	61.0	6.3	67.3
	40 " "	7	44.3	38.8	46.4	5.9	52.3
	20 I.U. vitamin A*	3	90	32	13.0	8.5	21.5
	40 " " "	7	49.4	48	33.5	5.9	39.4
Low vitamin A + 1 mg. desiccated thyroid per gm. body weight per day	60 $\gamma$ $\beta$ -carotene	3	29	22	110.5	3.3	113.8
	40 " "	7	33.3	37	89.4	3.1	92.5
	20 I.U. vitamin A	3	43	-8	7.1	2.2	9.3
	40 " " "	7	41.3	34	39.7	3.5	43.2
Low vitamin A with 0.5% thiourea	60 $\gamma$ $\beta$ -carotene	3	27	-8	21.3	1.0	22.3
	40 " "	4	26.2	14	3.6	0.93	4.5
	20 I.U. vitamin A	3	30	0	35.4	1.0	36.4
	40 " " "	4	26.5	9.2	52.2	0.87	53.1
Low vitamin A with 0.5% thiourea + 5 $\gamma$ DL-thyroxine per day	40 $\gamma$ $\beta$ -carotene	4	33.7	37.5	39.9	1.9	41.8
	40 I.U. vitamin A	4	26.2	44.2	43.1	1.7	44.8
Low vitamin A with 0.1% thiouracil in drinking water	40 $\gamma$ $\beta$ -carotene	7	42.8	12	6.3	1.6	7.9
	40 I.U. vitamin A	8	42.9	14	58.3	1.8	60.1
As above + 5 $\gamma$ DL-thyroxine per day	40 $\gamma$ $\beta$ -carotene	6	46.5	31.5	50.1	2.3	52.4
	40 I.U. vitamin A	7	43.4	29	53	2.0	55.0

\* Animals fed supplements of either 60  $\gamma$  of  $\beta$ -carotene or 20 I.U. of vitamin A per day were obtained from the stock colony and served as preliminary experiment. See text.

The growth rates of the animals were even more sensitive to excess thyroid or to the thio compounds during the period of supplementation with carotene or vitamin A than during the period of depletion. The groups fed thiourea or thiouracil gained an average of only 12 and 13 gm. respectively in 15 days, as compared with 47 gm. for the control group.

The failure to gain weight as a result of feeding thiourea was completely counteracted by thyroxine. With thiouracil, however, thyroxine only partly overcame the poor growth response. The animals receiving thyroid gained an average of 35 gm. in 15 days. There was no marked difference in the growth rate of the hypothyroid rats fed  $\beta$ -carotene and those fed vitamin A. The animals apparently metabolized enough of the carotene to meet the demands of such growth as was permitted by the state of decreased thyroid function. There were no eye lesions observed in any of the rats receiving either thiourea or thiouracil. Canadell and Valdecasas, however, have reported the development of xerophthalmia in rats fed carotene and rendered hypothyroid by the feeding of 2-methylthiouracil (20).

When either 40 i.u. of vitamin A or 40  $\gamma$  of  $\beta$ -carotene were fed daily to the control animals, roughly similar amounts of vitamin A accumulated in the liver and kidneys (total stores of 52.3 and 39.4  $\gamma$  respectively, Table II). The hyperthyroid rats fed vitamin A stored a similar amount, 43.2  $\gamma$ , but when such rats were given  $\beta$ -carotene, the storage of vitamin A was significantly greater than that in the control rats. Individual animals stored up to 130  $\gamma$  of vitamin A, and the average storage in the hyperthyroid rats fed carotene was 92.5  $\gamma$ .

The opposite effect was seen when the thyroid function was depressed by the administration of the thiourea and thiouracil. Such rats contained very little vitamin A as a result of feeding  $\beta$ -carotene, 4.5 and 7.9  $\gamma$  for thiourea and thiouracil respectively, as compared to 52.3  $\gamma$  in the control rats. That the livers and kidneys of such rats were still capable of storing available vitamin A was demonstrated by the comparatively high amounts of the vitamin found when the preformed vitamin had been fed along with the thio compounds, 53.1 and 60.1  $\gamma$  for thiourea and thiouracil respectively. Thus it appeared that the chief effect of an altered thyroid metabolism was upon the amount of vitamin A formed from carotene rather than upon the efficiency with which vitamin A was retained in the body.

Inasmuch as the hypothyroidism in the preceding experiments had been induced by drugs, it appeared desirable to determine whether the effects on vitamin A storage were a result primarily of the impairment of thyroid function or were due to some other pharmacological action of these compounds. Accordingly, sufficient thyroxine was administered to some of the animals receiving thiourea or thiouracil to approximate as nearly as possible a state of normal thyroid activity. When the hypothyroid condition was thus overcome, the animals fed  $\beta$ -carotene stored approximately the same amount of vitamin A as the control groups, 41.8 and 52.4  $\gamma$ , as compared with 39.4  $\gamma$  (Table I). The decreased conversion of carotene to vitamin A noted previously in rats fed thiourea and

thiouracil was therefore attributed to depressed thyroid function *per se* rather than to some extraneous effect of the compounds. They did, however, exert a specific effect that was not corrected with thyroxine. The amounts of vitamin A found in the kidneys were always very low in rats fed either of the thio compounds (Table II). This effect was seen whether  $\beta$ -carotene or vitamin A was fed, and it was not altered by the administration of thyroxine.

The question arose whether the observed effect of thyroid upon carotene metabolism was due to an increased basal metabolic rate or whether a more specific effect of thyroid was involved. 2,4-Dinitrophenol given orally or parenterally has been shown by several workers to produce an increased basal metabolic rate in experimental animals (21-23), although in many other respects its effects differ from those of the thyroid gland

TABLE III  
*Conversion of Carotene into Vitamin A by Rats Injected  
with Dinitrophenol (DNP)*

Low vitamin A diet plus	No. of rats	Mean body stores of vitamin A in liver and kidney
		$\gamma$
40 $\gamma$ $\beta$ -carotene per day	4	43.1 (29.9-48.2)*
40 i.v. vitamin A per day	5	54.1 (45.4-64.1)
40 $\gamma$ $\beta$ -carotene + 15 mg. DNP per kilo body weight per day	4	40 (28.0-45.3)
40 i.v. vitamin A + 15 mg. DNP per kilo body weight per day	4	45.4 (40.2-59.8)

\* The figures in parentheses are the ranges of values within the group.

(24). It thus offers a means of studying carotene metabolism under the influence of an increased metabolism, but without all of the general alterations that accompany an increase in the thyroid principle.

Accordingly, an experiment similar to those previously described was conducted with weanling rats from our stock colony. One group served as controls, the other group received a single daily subcutaneous injection of 6 mg. of 2,4-dinitrophenol per kilo of body weight during the depletion period. The dosage was increased to 15 mg. of the drug per kilo of body weight per day during the 15 days of vitamin supplementation. This dosage is in excess of the amount shown to bring about a continued basal metabolic rate of +30 to +50 per cent (25). The dinitrophenol was dissolved in water with the aid of half its weight of  $\text{NaHCO}_3$ , prepared in a concentration such that the desired daily dose was administered in 0.2 to 0.3 ml. of solution. When measurements were made 24 hours after the

last injection on rats that had previously received daily injections of the drug for 10 to 16 days, the oxygen consumption was 32 per cent above the control values. The dinitrophenol had no apparent effect on the ability of the animal to store vitamin A (Table III). More interesting, however, was the observation that the increased metabolic rate resulting from dinitrophenol injections was not accompanied by an altered carotene metabolism, as shown by the fact that the vitamin A stores in these animals were equal to those of control animals fed similar amounts of carotene (Table III). These results are in line with those of Greaves and Schmidt (26) who reported that the administration of thyroxine to experimental animals hastened the depletion of vitamin A reserves, but that dinitrophenol had no such effect.

#### DISCUSSION

Numerous authors have suggested that the thyroid gland is involved in vitamin A metabolism, and the general impression seems to be that the administration of thyroxine increases the need for the vitamin (10, 26, 27). Not all of the evidence, however, is in harmony with this conclusion (28), and the present experiments likewise suggest that the effect of thyroxine on preformed vitamin A is relatively minor. Rats fed halibut liver oil and enough thyroid tissue to increase the basal metabolic rate by 77 per cent contained at least as much vitamin A as control rats, 42.2 and 39.4  $\gamma$  respectively. In the presence of the thiourea and thiouracil, on the other hand, vitamin A retention was increased somewhat, total storages of 53.1 and 60.1  $\gamma$  versus 39.4  $\gamma$  in the controls. At least part of this increased retention, however, appears to be due to the fact that the rats fed the thio compounds were smaller than the controls and hence the standard doses of halibut oil fed were concentrated in a smaller amount of tissue. Unpublished data suggest that for the retention of vitamin A the size of the rat is at least as important as the basal rate.

On the other hand, the administration of desiccated thyroid tissue greatly increased the amounts of vitamin A stored in the body when carotene was fed. This increase in storage, in spite of a possibly increased requirement for the vitamin, leads to the conclusion that more vitamin A is formed from carotene in the presence of thyroid than in its absence. This might be brought about by a better absorption of carotene in the hyperthyroid rat or by an effect on the enzyme responsible for the conversion of carotene to vitamin A. In any event, the unequal amounts of vitamin formed from the same amount of carotene weaken the justification for using a provitamin as the international standard for vitamin A activity. In the hypothyroid rat very little vitamin A is produced from 0.6  $\gamma$



of  $\beta$ -carotene; in the hyperthyroid rat the amount may be twice that produced by the normal rat.

The present results seem to parallel the older observations that the livers of human beings suffering from hyperthyroidism often show an increased content of vitamin A. Moore reported that in four cases of exophthalmic goiter the liver contained 180, 300, 375, and 375 i.u. of vitamin A per gm., as compared to a median reserve of 220 i.u. per gm. for a large group of cases of accidental death (29). In a survey by Wolff (30) the means were 210 for exophthalmic goiter and 147 for accidental death. This increase in the storage of vitamin A in the hyperthyroid cases might be expected if the patients were deriving an appreciable part of their vitamin A from carotene.

#### SUMMARY

1. Weanling rats were depleted of vitamin A and at the same time were rendered either hyperthyroid by feeding desiccated thyroid tissue, or hypothyroid by feeding thiourea or thiouracil. They were then given either 40  $\gamma$  of  $\beta$ -carotene or 40 i.u. of vitamin A daily for 15 days, and the resulting stores of vitamin A in the liver and kidneys were determined colorimetrically.

2. Within the dosage limits employed, the ability to store preformed vitamin A was approximately the same in the hypo- or the hyperthyroid rats as in normal rats.

3. When carotene was fed, the hyperthyroid animals accumulated larger stores of vitamin A than normal rats receiving equivalent amounts of carotene. Rats receiving thiourea or thiouracil, on the other hand, stored very little vitamin A. The administration of thyroxine neutralized the effects of both thiourea and thiouracil, and restored the ability of the animals to convert carotene to vitamin A.

4. An increase in metabolic activity as a result of administering 2,4-dinitrophenol was not accompanied by an increased rate of conversion of carotene into vitamin A. It is therefore suggested that the altered carotene metabolism associated with thyroid dysfunction is not due to changes in the basal metabolic rate *per se*, but is brought about by some other physiological action of the thyroid gland.

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# INFRA-RED ABSORPTION SPECTRA OF STEROIDS

## IV. ADRENAL CORTICAL HORMONES AND RELATED STEROIDS\*

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All but one of the seven steroids studied in the present investigation may be classed as  $\Delta^4$ -pregnene derivatives containing a conjugated  $C_3$  ketone, an unconjugated  $C_{20}$  ketone, and either a hydroxyl or acetyl group at  $C_{21}$ . The exception is adrenosterone which has a ketone group rather than a side chain at  $C_{17}$ . Three of the compounds have a  $C_{11}$  ketone and two a  $C_{11}$  hydroxyl group. In this, as in previous papers of this series (1-3), the absorption spectra of the compounds under investigation will be discussed in relation to chemical structure, special attention being given to those structural configurations noted above.

### Methods

Absorption spectra were obtained with the Hardy infra-red spectrophotometer, with the use of the techniques employed in Paper I (1). All of the compounds were investigated in the form of solid films deposited on rock salt plates by methods previously described (1).

### EXPERIMENTAL<sup>1</sup>

The absorption spectra of the following adrenal cortical hormones and related steroids were studied: corticosterone (natural and synthetic), corticosterone acetate, desoxycorticosterone, desoxycorticosterone acetate (Fig. 1); 11-dehydrocorticosterone, 17-hydroxy-11-dehydrocorticosterone, and adrenosterone (Fig. 2). The spectrum of corticosterone in Fig. 1 was obtained with a sample isolated from adrenal cortex. The spectrum obtained with a sample of synthetic corticosterone was identical with that

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<sup>1</sup> We wish to express our gratitude to the following for donating crystalline samples of the steroids studied in this work: Dr. E. C. Kendall of the Mayo Clinic for natural corticosterone (Compound B), corticosterone acetate, adrenosterone, 11-dehydrocorticosterone (Compound A), and 17-hydroxy-11-dehydrocorticosterone (Compound E); Dr. Erwin Schwenk of the Schering Corporation for desoxycorticosterone and desoxycorticosterone acetate; and Dr. R. T. Major of Merck and Company, Inc., for synthetic corticosterone.

of the natural corticosterone with regard to wave-lengths and relative intensities of all absorption bands.

### *Analysis of Spectra*

*O—H, C—H, and CH<sub>3</sub> Absorption*—The compounds containing hydroxyl groups give absorption bands as a result of vibration of the O—H linkages at the following wave-lengths: corticosterone 2.79  $\mu$ , corticosterone acetate 2.91  $\mu$ , 11-dehydrocorticosterone 2.90  $\mu$ , 17-hydroxy-11-dehydrocorticosterone 2.86  $\mu$ , and desoxycorticosterone 2.87  $\mu$ . These wave-lengths are in the same range in which hydroxyl-containing androgens and pregnane derivatives were found to give O—H bands (1, 3). (For a discussion of variations in wave-lengths of O—H bands of steroids and the relation of hydrogen bonding to these variations see Paper I of this series (1).)

The fact that corticosterone acetate gives an O—H band is of interest, since at one time there was some uncertainty as to what kind of "unreactive" group contained the 4th oxygen of corticosterone (*e.g.*, see the review by Kuizenga (4)). Had an infra-red analysis of corticosterone acetate been made at the time, it would have been an easy matter to classify the "unreactive" group as hydroxyl.

As would be expected from our previous studies, all of the spectra show a strong linear C—H vibration band near 3.35  $\mu$ . They show two bands each in the 6.75 to 7.00  $\mu$  region, which may be ascribed to the angular vibration of "aliphatic" C—H linkages. Also, in all of the spectra, a band, which may be ascribed to methyl group vibration, occurs in the range 7.19 to 7.25  $\mu$ .

*C=O Absorption*—All of the compounds under study have a C<sub>3</sub> ketone conjugated with a  $\Delta^4$  double bond, and all give a band at 5.97 to 5.98  $\mu$ . This is in good agreement with the wave-length of absorption (5.97  $\mu$ ) of conjugated C<sub>3</sub> ketones encountered in steroids previously studied (1, 3).

An unconjugated C<sub>20</sub> ketone occurs in all of the compounds except adrenosterone. In a previous study of pregnane derivatives (3) it was found that C<sub>20</sub> ketone groups absorb in the range 5.84 to 5.87  $\mu$ . This is further substantiated in the present study by the occurrence of bands in this range in the spectra of corticosterone, corticosterone acetate, desoxycorticosterone, and desoxycorticosterone acetate. It will be noted that no absorption maxima occur in this range in the spectra of 11-dehydrocorticosterone and 17-hydroxy-11-dehydrocorticosterone. However, unpublished data from our laboratory indicate that in the spectra of these two compounds the C<sub>20</sub> ketone absorption actually occurs in this wave-length range, but is unresolved because of the masking effect of the strong absorption of the C<sub>11</sub> ketone and the conjugated C<sub>3</sub> ketone groups which are also present.

The absorption band of the unconjugated C<sub>11</sub> ketone is seen to occur at about 5.79  $\mu$  in the spectra of 11-dehydrocorticosterone and 17-hydroxy-

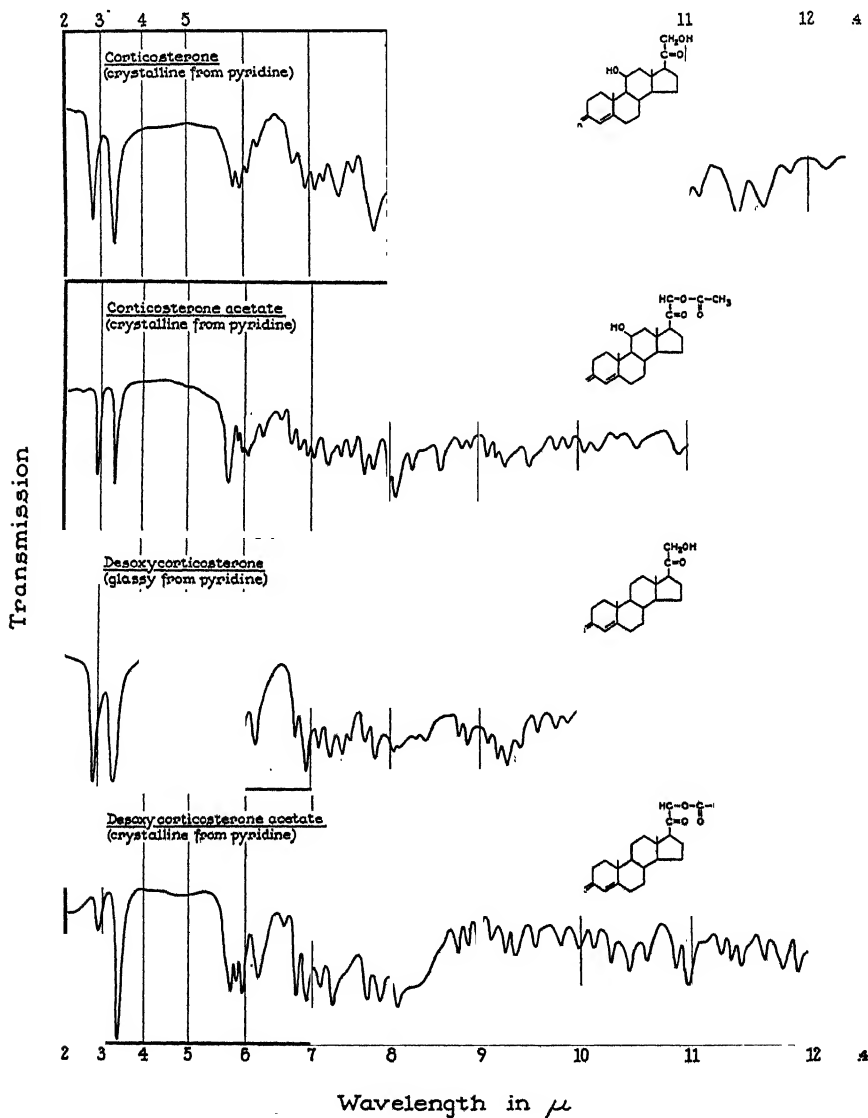


FIG. 1

11-dehydrocorticosterone. This wave-length is significantly higher than the wave-lengths (near 5.75  $\mu$ ) at which unconjugated C<sub>3</sub> and C<sub>17</sub> ketone

groups were reported to give absorption bands (1). However, in the spectrum of adrenosterone, the  $C_{11}$  ketone is unresolved, since the absorption bands resulting from the  $C_{11}$  and  $C_{17}$  ketone groups merge to give one large band with an absorption maximum near  $5.76 \mu$ .

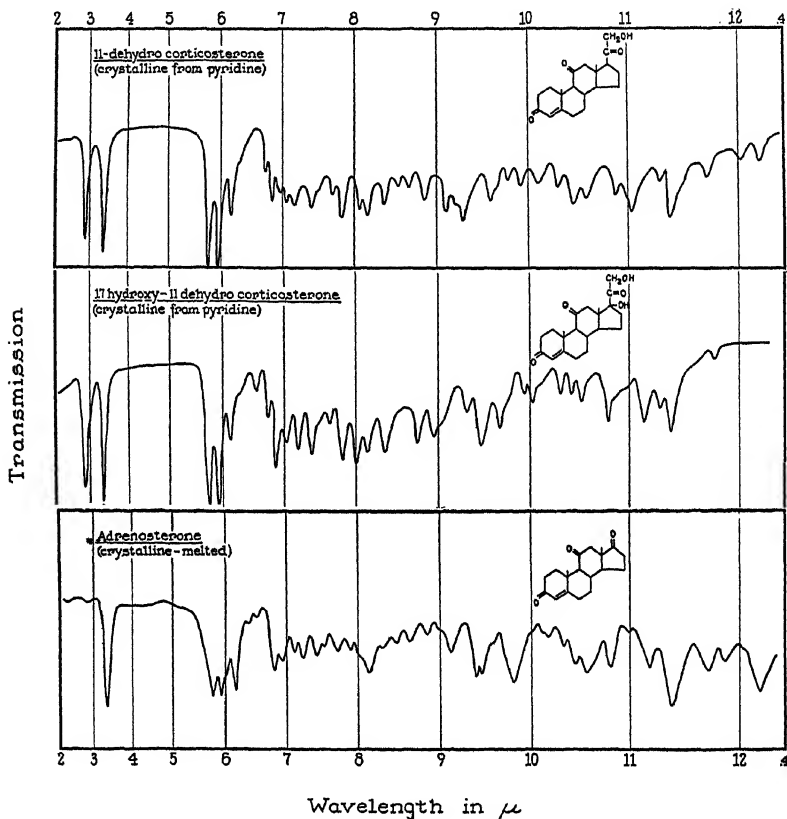


FIG. 2

The final type of carbonyl group encountered in the present series is that of an ester linkage. Such carbonyl groups occur in desoxycorticosterone acetate and corticosterone acetate and produce the bands at  $5.73 \mu$  in the spectra of both of these compounds. This is in good agreement with the  $5.75 \mu$  bands attributed to certain unconjugated ester carbonyls in spectra previously reported (1, 3).

**$C=C$  Absorption**—All of the steroids under study have a  $\Delta^4$  double

bond conjugated with a  $C_3$  ketone. On the basis of our earlier work (1, 3), such a conjugated double bond would be expected to give a band of medium to strong intensity near  $6.20 \mu$ . This band occurs in the spectra of adrenosterone ( $6.19 \mu$ ), 11-dehydrocorticosterone ( $6.19 \mu$ ), 17-hydroxy-11-dehydrocorticosterone ( $6.15 \mu$ ), desoxycorticosterone ( $6.21 \mu$ ), and desoxycorticosterone acetate ( $6.21 \mu$ ). However, the spectra of corticosterone and corticosterone acetate differ from the spectra of the compounds listed above in that they do not show a single strong band in the conjugated  $C=C$  absorption region. Corticosterone gives a band of medium intensity at  $6.07 \mu$  and one of weak intensity at  $6.24 \mu$ , while corticosterone acetate gives a band of medium intensity at  $6.05 \mu$  and a band of weak intensity at  $6.31 \mu$ . The reason for the variations is not at present apparent.

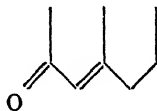
*C—O Absorption*—The most commonly occurring C—O group in the present series is that of the  $C_{21}$  hydroxyl group, which is present in desoxycorticosterone, corticosterone, 11-dehydrocorticosterone, and 17-hydroxy-11-dehydrocorticosterone. The spectra of all of these compounds show a band of medium to strong intensity in the narrow range of  $9.28$  to  $9.32 \mu$ . These bands may arise from the C—O vibration of a  $C_{21}$  hydroxyl adjacent to a  $C_{20}$  ketone; however, the present experimental evidence for this inference is admittedly incomplete. Of the four compounds with  $C_{21}$  hydroxyls, corticosterone gives the strongest absorption band in the  $9.28$  to  $9.32 \mu$  region. This may possibly result from a merging of the C—O absorption of both the  $C_{21}$  and  $C_{11}$  hydroxyl groups of corticosterone. Should this be the case, it would follow that the band at  $9.27 \mu$  in the spectrum of corticosterone acetate arises from the C—O absorption of the  $C_{11}$  hydroxyl. Adrenosterone and desoxycorticosterone acetate, neither of which has a  $C_{21}$  hydroxyl, give no band in the  $9.28$  to  $9.32 \mu$  region.

It should be reemphasized that the assignment of wave-length regions to absorption by C—O linkages in specific hydroxyl groups is of a tentative nature, but certain possible relationships have been given above, which may serve as a basis for future study. In the case of the C—O of ester linkages, in which the carbon has one double bond attached to it, the assignment of absorption bands is on a much firmer basis (1, 3, 5). There is little doubt that the large bands near  $8.05 \mu$  in the spectra of corticosterone acetate and desoxycorticosterone acetate arise from the ester C—O linkages of these compounds.

*Other Absorption Bands*—In the spectra of  $\Delta^4$ -androstenedione-3,17, testosterone, testosterone propionate (1), progesterone (3), and  $\Delta^4$ -cholestenone (unpublished results), a major absorption band occurs in the region  $11.49$  to  $11.57 \mu$ . This band appears to arise from some vibration in structural configuration (A), which is common to all of these molecules.



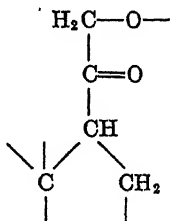
Since this configuration is present in all the compounds of the present series, it is of considerable interest to investigate their spectra with relation to bands in the region around  $11.5 \mu$ .



(A)

The major bands in this region for these compounds are as follows: desoxycorticosterone  $11.52 \mu$ , desoxycorticosterone acetate  $11.42 \mu$ , corticosterone  $11.43 \mu$ , corticosterone acetate  $11.37 \mu$ , 11-dehydrocorticosterone  $11.40 \mu$ , 17-hydroxy-11-dehydrocorticosterone  $11.40 \mu$ , adrenosterone  $11.40 \mu$ . It would therefore appear that whenever there is a hydroxyl or ketone group at  $C_{11}$  the presence of structural configuration (A) gives rise to a band in the region  $11.37$  to  $11.43 \mu$ . Desoxycorticosterone, with no oxygen at  $C_{11}$ , gives the band in the  $11.49$  to  $11.57 \mu$  region. Desoxycorticosterone acetate, however, gives a band at  $11.42 \mu$ , despite the fact that it has no oxygen at  $C_{11}$ . This discrepancy may possibly have its explanation in some effect exerted by the acetate group at  $C_{21}$ . Some evidence for such an effect is obtained from the fact that the band in question in corticosterone acetate ( $11.37 \mu$ ) is also shifted to a lower wave-length than that given by unacetylated corticosterone ( $11.43 \mu$ ).

A comparison of the spectra presented in this paper also reveals that five of the compounds give a strong absorption band near  $11 \mu$ . These are corticosterone ( $10.99 \mu$ ), desoxycorticosterone ( $11.00 \mu$ ), 11-dehydrocorticosterone ( $11.03 \mu$ ), corticosterone acetate ( $10.92 \mu$ ), and desoxycorticosterone acetate ( $10.96 \mu$ ). All of these compounds contain structural configuration (B). Adrenosterone and 17-hydroxy-11-dehydrocorticosterone, which do not contain configuration (B), do not give a strong band near  $11 \mu$ . It therefore appears likely that this configuration is responsible for the bands mentioned above, which occur in the range of  $10.92$  to  $11.03 \mu$ .



(B)

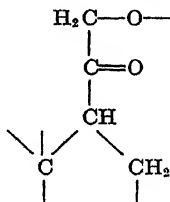
## SUMMARY

1. The infra-red absorption spectra, from 2 to 12.4  $\mu$ , of a number of adrenal cortical hormones and related steroids have been presented.

2. Certain absorption bands in the spectra have been discussed in relation to the chemical structure of these compounds.

3.  $C_{11}$  ketone groups give rise to a strong absorption band with a maximum near 5.79  $\mu$ .

4. The spectra of corticosteroids containing the structural configuration



have a characteristic strong absorption band in the region of 10.92 to 11.03  $\mu$ .

5. The absorption spectra of natural and synthetic corticosterone are identical.

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# AN ELECTROPHORETIC STUDY OF THE EGG WHITE PROTEINS OF VARIOUS BIRDS\*

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The ovalbumins of chickens, turkeys, guinea-hens, ducks, and geese show varying immunological similarities, although it is possible to distinguish the five albumins with selected sera against chicken ovalbumin (1). Furthermore, mixtures of two albumins, such as guinea-hen and duck ovalbumin, can be clearly distinguished electrophoretically (2).

Marked specificities are shown by the electrophoretic diagrams of the blood serum (3) and plasma proteins (4) as well as by the milk whey proteins (5) of various animals. Such previous findings prompted an investigation of the proteins of the egg white of a number of birds. It was found that the egg white proteins of each species can be identified by their electrophoretic properties, although only relatively slight differences are observed between species in which previous studies have shown that the ovalbumins are closely related immunologically.

## EXPERIMENTAL

The egg whites of the various species studied were separated as thoroughly as possible from the yolks, extreme care being taken to keep the vitellin membrane intact. 3 volumes of diethyl barbiturate buffer of pH 8.6 and ionic strength of 0.1 were added to the egg white, and the solutions were stirred so that all of the clumps were dispersed. Small amounts of mucous strands and the chalaza were then removed by centrifugation. The clear supernatant solutions were dialyzed from 48 to 96 hours at 0° against several changes of buffer and were analyzed electrophoretically. The duration of the electrophoretic experiments was 10,800 seconds with a constant potential gradient of between 5.8 and 6.3 volts per cm. All analytical measurements were made on the descending patterns. In so far as it was possible, the various electrophoretic components were designated according to the terminology used by Longworth, Cannan, and MacInnes (6) for the chicken.

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In this respect the slowest of the well defined components possessing a negative mobility in the buffer used have been termed conalbumins and their electrophoretic areas designated as  $C_1$  and  $C_2$  in order of their decreasing mobilities, as is shown in Fig. 1 for the chicken egg white. If this area is made up of only one component, it is designated  $C$ . In many of the egg whites examined, there appeared varying amounts of protein which migrated between the conalbumins and the salt boundary. Such slowly migrating material is indicated in the electrophoretic diagrams by an  $S$ . In most instances it is poorly defined electrophoretically and often is closely associated with the salt boundaries. For this reason no mobilities have been given for this area.

The electrophoretic components between the conalbumins and albumins have been referred to as globulins and designated as  $G$ . Included in this region is probably the ovomucoid. This region was relatively heterogeneous electrophoretically in most of the egg whites examined, and again no mobilities are reported for this area.

The components of higher mobility, which in chicken egg white comprise the major portions of the proteins and are known to represent the albumin, have been designated as  $A$ . Thus in Fig. 1 the areas designated as  $A_1$  and  $A_2$  represent two albumins closely related in mobility. A small amount of an electrophoretic component which has a higher mobility than the albumin but resolves well from these proteins has been referred to as a "fast" component and designated  $F$  in this diagram (Fig. 1). When such protein material was present in any egg white in amounts less than 1 per cent of the total proteins, its area was included with the albumin.

The chicken, turkey, guinea hen, pheasant, mallard duck, and goose showed the presence of small amounts (0.2 to 1.5 per cent) of a protein isoelectric above pH 8.6, and hence with a positive electrophoretic mobility in the buffer used. No attempt was made to include this component as a part of the analytical data. Its presence in any noticeable amount is indicated by asymmetries in the ascending salt boundary.

The interaction of oppositely charged protein molecules in electrophoretic experiments has been noted previously (6-8). In the egg whites containing this protein of high isoelectric point, it is likely that the mobilities of the proteins having a negative net charge in the buffer used are lowered somewhat by interaction with the above component. Longworth, Cannan, and MacInnes (6) have previously referred to this component in the egg white of the chicken as  $G_1$  (globulin).

The marked variations in the electrophoretic patterns of the species studied make any such nomenclature as the above arbitrary until the several components have been separated and characterized. However, the system of numbering components used in this study will serve a refer-

ence function until a separation of the proteins in these egg white systems has been accomplished. In the discussion of the experimental results, the electrophoretic patterns of the various species will be discussed, with the chicken pattern as the basis of comparison.

### Results

*Chicken (White Leghorn)*—The electrophoretic pattern of the egg white of this species is shown in Fig. 1. This figure is quite similar in appearance to the diagram obtained by Longworth, Cannan, and MacInnes (6) at pH 8.0, except for the somewhat better resolution that would be expected at a higher pH. In this and other patterns no attempt has been made to designate individual components in the relatively heterogeneous area labeled *G* (globulins). A small amount of the relatively heterogeneous

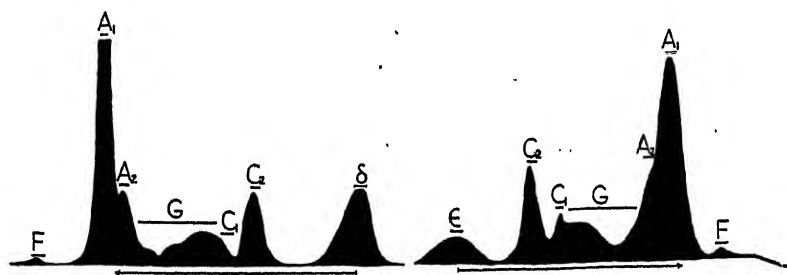


FIG. 1. Electrophoretic diagram of chicken egg white proteins

*F* component is present. Analytical data for all species studied are shown in Table I.

*Turkey (Bronze)*—From Fig. 2 it can be seen that the electrophoretic components of turkey egg white closely resemble those of the chicken. The conalbumins have somewhat higher electrophoretic mobilities and comprise a smaller per cent of the total egg white proteins, as compared to those in chicken egg white. The area immediately following *C*<sub>2</sub> is, however, noticeably different. Furthermore the *A*<sub>2</sub> and *A*<sub>1</sub> components are not as well resolved, and, as shown in Table I, the analytical data differ somewhat from those for the chicken.

*Guinea Hen*—As seen from Fig. 3, this species shows marked deviations from the chicken in the electrophoretic diagram of its egg white proteins. As in the case of turkey egg white, the conalbumins have slightly higher mobilities than those of the chicken egg white. A larger amount of the *C*<sub>1</sub> component is present and the globulin area does not extend over as wide a mobility range as the analogous area of chicken egg white. A much higher ratio of the area *A*<sub>2</sub> to *A*<sub>1</sub> is another distinguishing feature.

*Pheasant (Ring-Neck)*—A further departure from the electrophoretic pattern of chicken egg white is shown by the egg white of this species (Fig. 4). Three proteins of closely related electric charge occupy the area

TABLE I  
*Electrophoretic Data for Egg White Proteins of Various Bird Species*

Species	Component														
	S		C <sub>3</sub>		C <sub>2</sub>		C <sub>1</sub>		G	A <sub>2</sub>		A <sub>1</sub>		F	
	per cent	μ*	per cent	μ*	per cent	μ*	per cent	μ*	per cent	μ*	per cent	μ*	per cent	μ*	
Chicken					16.0	1.8	6.5	2.6	15.0	8.5	4.8	52.0	5.5	2.0	6.9
Turkey	0.5				13.0	2.2	3.0	2.8	14.0	18.0	4.7	50.5	5.5	1.0	7.4
Guinea hen	0.8				18.4	2.3	10.4	2.9	15.9	19.9	4.8	33.6	5.5	1.0	6.9
Pheasant	1.5				11.0	2.2	6.0	2.6	14.5	54.0	4.8	11.5	5.6	1.5	7.2
Goose	1.0	1.5	2.0		7.0	2.5	4.0	2.8	29.0	33.5	6.1	22.0	6.7	2.0	
Mallard duck	2.5				5.0	2.4	6.5	2.8	19.5	31.0	5.8	34.5	6.5	1.0	7.6
Muscovy "	3.5				1.5	2.4	9.0	2.8	21.5	17.0	5.4	45.5	6.1	2.0	7.3
American coot	2.0	8.0	1.3		4.5	2.0	3.5	2.3	16.0	27.0	4.5	27.0	5.1	12.0	6.2
Black tern	0.6				7.9	1.4	5.5	2.6	14.8	66.2	6.0	5.0	7.2		
Common pigeon	0.5				11.0	2.1			48.0		35.0		4.9	5.5	6.4
Ringed turtle-dove	1.0				14.0	2.7			50.0		35.0		6.0		
Dove hybrid	2.9				9.5	2.4	3.7	2.8	50.5		33.4		5.8		
English sparrow	1.0				8.0	2.0	10.0	2.3			81.0				

\* Mobilities expressed as  $\text{cm}^2 \text{ volt}^{-1} \text{ sec}^{-1} \times 10^6$ .

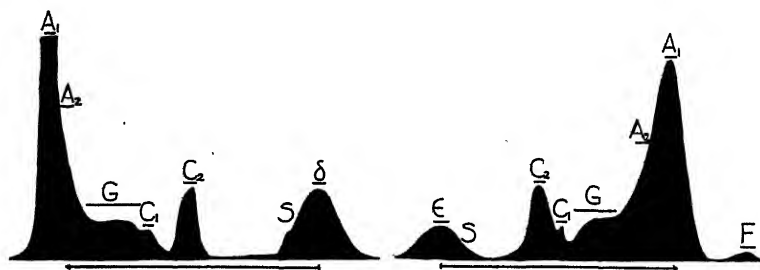


FIG. 2. Electrophoretic diagram of turkey egg white proteins

possessing the approximate mobility of ovalbumin. The slower of these three proteins has been designated *G*, since the other two components possess electrophoretic mobilities analogous to the *A*<sub>1</sub> and *A*<sub>2</sub> components

of the chicken. A conalbumin area showing only slight electrophoretic resolution and a larger amount of the *S* component further distinguishes the egg white proteins of this species. Inspection of the analytical data of Table I reveals this variation.

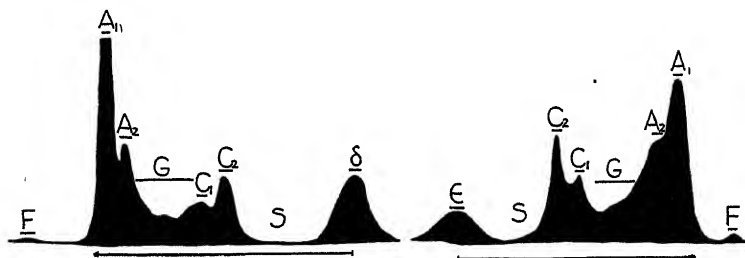


FIG. 3. Electrophoretic diagram of guinea hen egg white proteins

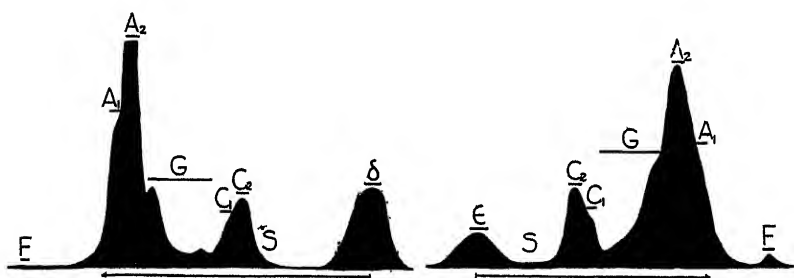


FIG. 4. Electrophoretic diagram of pheasant egg white proteins

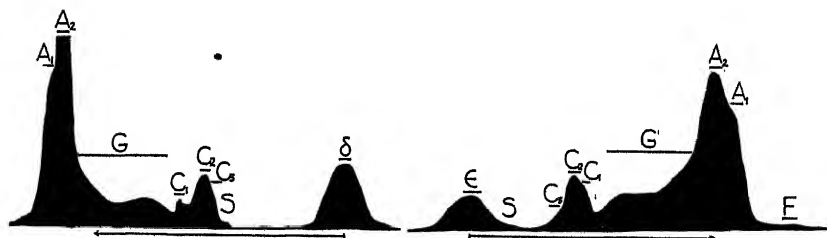


FIG. 5. Electrophoretic diagram of goose egg white proteins

*Goose (Toulouse)*—In Fig. 5, the electrophoretic patterns of the egg white proteins of the goose reveal a closely related complex of proteins in the conalbumin area. The two albumins possess higher mobilities than is the case for the analogous chicken egg white proteins. In addition, the



$A_2$  component is present in larger amounts than the  $A_1$  component. A relatively heterogeneous globulin area, which resembles that of turkey egg white more closely than that of the chicken further characterizes this protein system.

*Duck (Mallard)*—The electrophoretic diagram of the egg white of this species (Fig. 6) is somewhat similar to that of the goose. However, there is more of the  $A_1$  than of the  $A_2$  component, and the material designated  $S$  is present in larger amounts than in the egg white systems of the species discussed above, making up some 2.5 per cent of the total proteins. In goose egg white, the  $A_2$  component predominates.

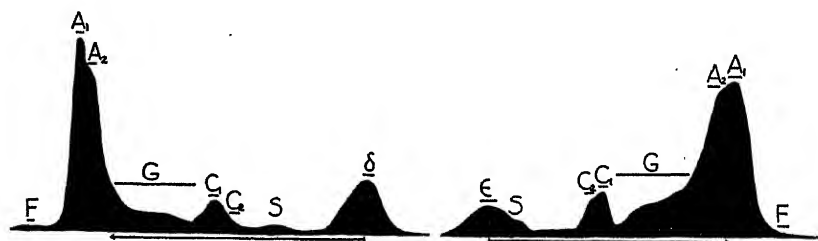


FIG. 6. Electrophoretic diagram of mallard duck egg white proteins

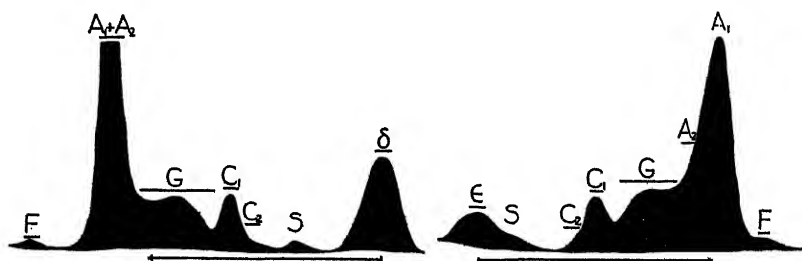


FIG. 7. Electrophoretic diagram of muscovy duck egg white proteins

*Duck (Muscovy)*—The electrophoretic pattern of the egg white of this species (Fig. 7) can be clearly distinguished from its close relative, the mallard duck. A larger amount of the heterogeneous  $S$  component and deviations tending toward more homogeneity in the conalbumin and albumin areas permit such a differentiation. With the exception of somewhat higher electrophoretic mobilities the pattern of the albumin and globulin areas closely resembles the analogous proteins of turkey egg white.

*American Coot (Mud Hen)*—The electrophoretic diagram for the egg white of this species is shown in Fig. 8. It differs markedly from the egg

white of the other water-fowl studied in all of the electrophoretic areas, particularly in the *F* component. Present in this egg white are three well defined conalbumins, two closely related albumins which are present in equal amounts, and a relatively large and well defined *F* component. The albumins have markedly lower mobilities as compared to those found in the egg whites of the other water-fowl; indeed, the *F* component has an electrophoretic mobility more closely related to the  $A_1$  than to the *F* component of the egg white of the other water-fowl.

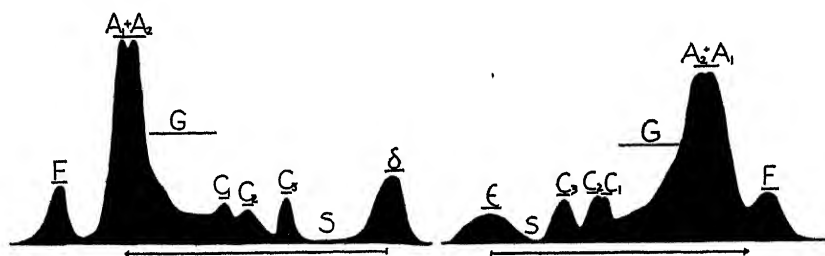


FIG. 8. Electrophoretic diagram of American coot egg white proteins

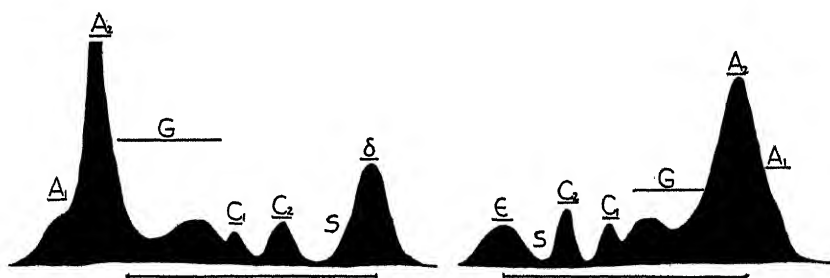


FIG. 9. Electrophoretic diagram of black tern egg white proteins

*Black Tern*—The electrophoretic pattern of the egg white proteins of the black tern is shown in Fig. 9. Two conalbumins are well resolved from a relatively heterogeneous globulin area which merges with the slower moving portion of the albumin area. The  $A_1$  component of the albumin area makes up only a small percentage of these proteins and has a mobility analogous to the *F* component in the egg white protein systems of the Galliformes species (chicken, turkey, guinea hen, and pheasant). Such data are reported in detail in Table I.

*Common Pigeon*—The electrophoretic pattern of pigeon egg white proteins (Fig. 10) and the patterns of the corresponding proteins of the

two types of dove and the English sparrow exhibit certain similarities. A well defined conalbumin is the only feature of the electrophoretic diagram of the pigeon egg white which is comparable to that found in the eggs of the species shown in Figs. 1 to 9 inclusive. The area designated *G* is the largest component and is more or less merged with the proteins of higher mobility which are designated as albumin (*A*). Unlike the previously discussed species, the egg albumin component of the egg white of the pigeon does not resolve into two components as seen from Table I, and makes up a smaller percentage of the total egg white proteins. A well defined *F* component is also present.

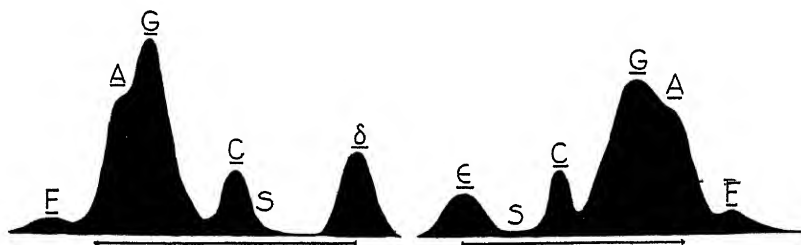


FIG. 10. Electrophoretic diagram of pigeon egg white proteins

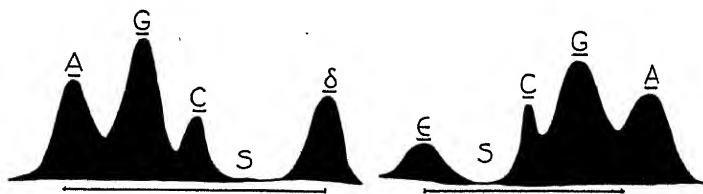


FIG. 11. Electrophoretic diagram of ringed turtle-dove egg white proteins

*Ringed Turtle-Dove*—The electrophoretic pattern of the egg white of this species (Fig. 11) shows a marked difference from that of the closely related common pigeon (Fig. 10). A distinguishing feature is the resolution of the *A* component from the *G* area. The mobility of the *A* component of the egg white of the ring dove approaches that of the *F* component of the pigeon. No analogous electrophoretic area is present in the egg white proteins of the ring dove.

*Hybrid Dove*—As judged by electrophoresis, the egg white of hybrids resulting from the mating of a male ringed turtle-dove (*Streptopelia risoria*) and a female oriental dove (*Streptopelia orientalis*) shows a marked similarity to that of the ring dove. However, as seen in Fig. 12, an additional small component designated *C*<sub>1</sub> appears on the shoulder of the *G*

component and a better resolution of the *G* and *A* regions is evident. Certain of these differences are revealed by a comparison of the analytical data as shown in Table I. The eggs of *Streptopelia orientalis* could not be obtained, but by inference it can be assumed that the electrophoretic patterns of the egg white of this species would show variations from the analogous proteins of the ring dove.

*English Sparrow*—The electrophoretic pattern of the egg white proteins of this species resembles that of the pigeon quite closely. In Fig. 13, however, it can be seen that the albumin portion of the closely related *G* area is more heterogeneous and is present in smaller amount than is true

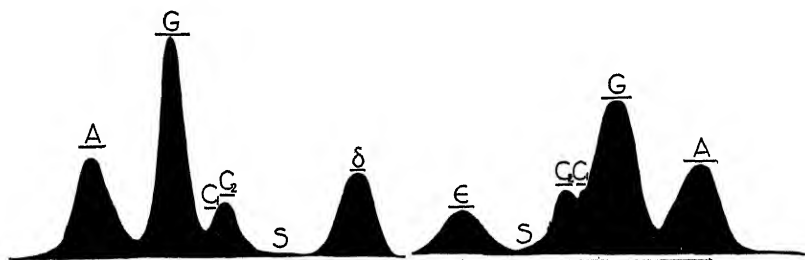


FIG. 12. Electrophoretic diagram of hybrid dove egg white proteins

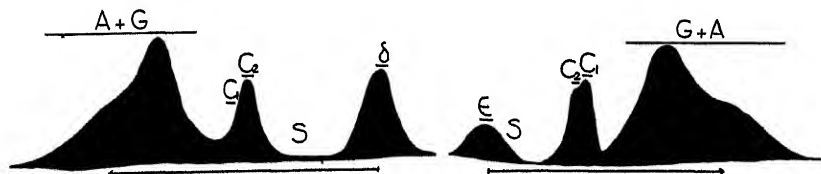


FIG. 13. Electrophoretic diagram of English sparrow egg white proteins

in the case of the pigeon egg white protein system. No attempt has been made in Table I to calculate mobilities or percentage composition of the components in this area. The conalbumins are made up of two closely related proteins. The salt boundaries suggest the presence of protein of low or of no net charge.

#### DISCUSSION

In this study the egg white proteins of thirteen species of birds belonging to six different phylogenetic orders were examined electrophoretically. A characteristic pattern was obtained for each species. There are, however, greater similarities for the species within a given order than for less closely related species. Thus, the Galliformes have egg white proteins relatively

closely related electrophoretically. The same is true for the Anseriformes (goose, mallard duck, and muscovy duck). The difference in mobilities of the ovalbumins of these two orders, as noted by Landsteiner *et al.* (2), with absorption methods for the location of the boundaries, is well illustrated in Table I, where it can be seen that, while the mobilities of the  $A_1$  and  $A_2$  components of the Galliformes species are identical, the same is not true for the Anseriformes species. In the latter order the analogous albumin components have higher electrophoretic mobilities than the corresponding proteins of the Galliformes and they differ for each of the species.

The three members of the order Columbiformes (common pigeon, ringed turtle-dove, and a hybrid of the latter species) showed marked similarities in the electrophoretic patterns of their egg white proteins, but the group as a whole showed patterns of pronounced difference from all other species studied, with the exception of the English sparrow. In this respect the chief points of difference of the Columbiformes species were the presence of a single conalbumin area, a very high percentage of the area designated as globulin, and the presence of a relatively low percentage of albumin. In the case of the common pigeon this latter area showed poor electrophoretic resolution from the globulin area. The ringed turtle-dove (*Streptopelia risoria*) could be distinguished from the hybrid of this and an oriental species (*Streptopelia orientalis*), even though the differences in the electrophoretic patterns were not great. Colovos (9) has shown that the sera of the common pigeon and the ring dove can be distinguished electrophoretically, although he did not examine the sera of the hybrid.

The egg white of the English sparrow (Passeriformes) showed a closer electrophoretic resemblance to that of the common pigeon than to any of the other species examined, although the presence of two conalbumins, closely related electrophoretically, and a more heterogeneous and closely related globulin-albumin area allows one to distinguish these two egg white systems easily.

The electrophoretic patterns of the egg whites of the American coot or mud hen (Gruiformes) and the black tern (Charadriiformes) show certain similarities to the corresponding diagrams of the Galliformes and Anseriformes species. The conalbumins of these two species are, however, better resolved electrophoretically and, in the case of the egg white of the mud hen, three well resolved components appear in the diagrams. The albumin proteins of the egg white of the mud hen have the lowest electrophoretic mobilities of any observed in this study.

Unfortunately, eggs were obtained for only one species of the orders Passeriformes, Gruiformes, and Charadriiformes. A further study of additional species in each of these or in other orders would allow one to determine whether similarities such as those noted for the Galliformes species are common to other orders as well. Such work is being initiated.

This limited electrophoresis study reveals the same type of species specificities of egg white proteins that has been found for the proteins of other biological fluids (3-5). The greatest similarities of the electrophoretic patterns appear in the conalbumin area usually designated  $C_2$ . In general, the areas designated as globulins were relatively heterogeneous. The albumins showed a relatively wide variation in mobilities and in percentage distribution into the two components designated as  $A_1$  and  $A_2$ . The egg whites of most of the species showed the presence of electrophoretically ill defined proteins which migrate at a rate which is slower than that of the conalbumins under comparable conditions. In addition, a small amount of a protein isoelectric above pH 8.6 was present in the egg whites of the chicken, turkey, guinea hen, mallard duck, pheasant, and goose.

The authors wish to thank Dr. M. R. Irwin for gifts of the eggs of the Columbiformes species and to acknowledge the interest of Dr. J. W. Williams in this work.

#### SUMMARY

The egg white proteins of thirteen species of birds were examined electrophoretically. It is possible to differentiate each species by this technique.

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IDENTIFICATION OF PREGNANE-3( $\alpha$ ),17-DIOL-20-ONE AS  
THE STEROID MOIETY OF A NEW GLUCURONIDE  
ISOLATED FROM HUMAN URINE

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In 1943 one of us (H. S. S.) and coworkers (1) reported the isolation of a new steroid glucuronide from the urine of a young female pseudohermaphrodite.<sup>1</sup> The free steroid was obtained by acid hydrolysis and was characterized tentatively as a steroid having an  $\alpha$ -OH at C-3 and a ketone group at C-20. Some further work was done on the steroid moiety in the laboratories of the School of Medicine, University of Pittsburgh, and Magee Hospital. Changing circumstances made it necessary for the original investigators to discontinue their study, and work on the identity of the steroid moiety was undertaken at the Mayo Foundation. This report covers the work done since the preliminary report.

Urine was processed by Venning's (2) method for the isolation of sodium pregnanediol glucuronide (NaPG). The glucuronide obtained, however, differed from NaPG in physical appearance and solubility. Analysis indicated the formula  $C_{27}H_{41}O_9Na$ , the sodium glucuronide of a steroid with the formula  $C_{21}H_{34}O_3$ . Reduction of Benedict's reagent after hydrolysis and a positive result of Tollens' naphthoresorcinol test indicated the presence of glucuronic acid.

Several preparations were hydrolyzed for isolation of the steroid moiety. In the earlier work only material that had been extensively recrystallized was used. An acetate was prepared by Dr. K. Hofmann from some of the ketonic steroid so obtained, which was shown to be identical with the acetate obtained later. In the later work a relatively crude preparation of glucuronide was used, and treatment of the steroid moiety with Girard's Reagent T gave some pregnanediol in addition to the ketone previously described. The analytical results suggest that the highly purified

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<sup>1</sup> Patient of Dr. John W. Shirer of the staff of the Elizabeth Steel Magee Hospital, to whom we are indebted for the opportunity to study the urine.



glucuronidate contained little if any NaPG, but the crude material must have contained an appreciable amount.

Enzymic and acid hydrolysis of the purified glucuronidate gave essentially the same results. In both instances approximately 25 per cent of the neutral fraction was non-ketonic in nature. The presence of this material may indicate a small amount of NaPG even in the purified glucuronidate. The principal steroid, however, was the ketone.

Analysis of the ketonic steroid indicated the formula  $C_{21}H_{34}O_3$ , and the results of analysis of the acetate agreed with its formulation as a monoacetate of  $C_{21}H_{34}O_3$ . In the meantime Lieberman and Dobriner (3) described the isolation from human urine of pregnane-3( $\alpha$ ), 17-diol-20-one and the preparation of its acetate. The close similarity in the properties of the steroid and its acetate obtained from the glucuronidate and those of pregnane-3( $\alpha$ ), 17-diol-20-one and its acetate, respectively, strongly indicated their identity. A sample of pregnane-3( $\alpha$ ), 17-diol-20-one furnished by Dr. Lieberman melted at 207–208°, and a sample of our material which melted at the same temperature did not depress the melting point of the Lieberman sample. However, the specific rotation of our material was  $[\alpha]_D^{28} = +51.8^\circ \pm 0.8^\circ$ , whereas Lieberman and Dobriner<sup>2</sup> gave  $[\alpha]_D^{28} = -7.8^\circ \pm 2.6^\circ$ . Nevertheless, it still seemed probable that the structure must be essentially that of pregnane-3( $\alpha$ ), 17-diol-20-one, although the presence of an 11( $\beta$ )-OH was by no means ruled out.

Oxidation of the acetate with chromic acid gave a neutral product which could not be induced to crystallize. Hydrolysis and crystallization from dilute acetone gave needles which did not depress the melting point of etiocholan-3( $\alpha$ )-ol-17-one. Conversion to the benzoate gave a product which melted at 160–162° and did not depress the melting point of etiocholan-3( $\alpha$ )-ol-17-one benzoate.<sup>3</sup> It was concluded, therefore, that the original ketone belonged to the cholane series, and that it possessed a hydroxyl group at C-3 with the  $\alpha$  configuration, a hydroxyl group at C-17, and, also at C-17, a 2-carbon side chain bearing the carbonyl group at C-20. This is the formulation of pregnane-3( $\alpha$ ), 17-diol-20-one.

The melting point of this ketone deserves comment. The specimen used for determination of the specific rotation melted at 212–213°. After evaporation of the alcoholic solution in a water bath the melting point was 190–195°. Recrystallization from ethyl acetate raised the melting

<sup>2</sup> In correspondence Dr. Lieberman has informed us that the published value is in error and that the correct value should be  $[\alpha]_D^{28} = +64.5^\circ \pm 4^\circ$  ( $c = 0.248$  in alcohol). We are still not in agreement on the exact value for the specific rotation, but the major discrepancy has been eliminated.

<sup>3</sup> The previous observation (1) that the Zimmermann reaction was typical of a 3-ketosteroid after oxidation with chromic acid may perhaps be explained by the use of too short a period of oxidation (1 hour) for the formation of appreciable amounts of the 17-ketosteroid.

point only to 198–200°, and it could not be raised above 200–203° by further recrystallization from acetone. Possibly there was conversion of a small amount to the D-homo compound (compare Hirschmann and Hirschmann (4)), although none of the treatments was more drastic than those used originally for isolation of the compound.

Since pregnane-3( $\alpha$ ),17,20-triol has been obtained from the urine of pseudohermaphrodites (5–7), this substance was sought in the non-ketonic fraction derived from the crude glucuronide. Chromatographic analysis, however, gave only pregnanediol.

Glucuronic acid or glucurone was not isolated after hydrolysis of the conjugate. However, the analysis and the development of reducing power on hydrolysis indicated the presence of a uronic acid which was presumed to be glucuronic acid, since urinary substances are commonly conjugated with that acid and pregnanediol is known to be so conjugated. The color given with naphthoresorcinol and enzymic hydrolysis of the conjugate with liver powder lend further support to the conclusion that the steroid was conjugated with glucuronic acid.

#### EXPERIMENTAL

*Isolation of Sodium Steroid Glucuronide*—Urine was collected in glass containers containing 5 drops of tricresol or tricresol and 100 ml. of butyl alcohol and was sent to the laboratory at Magee Hospital in lots of 2 to 3 liters. At times 2 to 3 weeks elapsed between collection of the specimens and processing on receipt at the laboratory. Collections were made periodically from May, 1940, to December, 1945. The urine was processed according to the method of Venning (2). The acetone from which the glucuronide was precipitated was evaporated, the residue was taken up in a small volume of alcohol, and 5 volumes of 0.1 N NaOH or water were added. No precipitable free steroid was found by the procedure of Astwood and Jones (8) in eleven different specimens. The amounts of crude sodium glucuronide obtained from various batches of urine varied from 33 to 79 mg. per liter.

The crude glucuronide contained some resinous material which was removed by treatment with cold alcohol. One batch of 2.4 gm. thus gave 1.6 gm. of partially purified material. By recrystallization twice from 95 per cent alcohol a first crop of 429 mg. and a second crop of 221 mg. were obtained. Both crops melted at 266–268° (4° per minute) with decomposition. Analysis of the second crop and of another preparation gave the following results after drying to constant weight at 107° in a high vacuum.

<i>Analysis</i> — $C_{27}H_{41}O_9Na$ . Calculated.		C 60.88, H 7.76, Na 4.32
Found.	" 60.25, " 7.77, "	" 4.26
"	" 60.46, " 7.80, "	" 4.27

Analysis for glucuronic acid by the method of Allen and Viergiver (9) gave titers similar to those obtained with an equal weight of NaPG. When the method Maughan, Evelyn, and Browne (10) (based on Tollens' naphthoresorcinol reaction) was used, the new substance gave slightly less color than an equal weight of NaPG, but only a small amount of color was extracted by ether, and the amount extracted was not proportional to the amount of substance used. On standing overnight there was considerable increase of color in the ether layer in proportion to the concentration of substance used. After acid hydrolysis, but not before, Benedict's solution was reduced. In the Zimmermann reaction the glucuronide gave a color similar to that of the free steroid (1) and its acetate.

*Hydrolysis of Crude Glucuronide*—The glucuronide (500 mg.) was dissolved in 10 ml. of alcohol and 10 ml. of water by warming. The solution was diluted to 100 ml., 50 ml. of toluene were added, and the mixture was heated to boiling. Refluxing was continued for 1 hour after cautious addition of 11 ml. of concentrated HCl. After cooling, the toluene was separated and the aqueous solution was extracted twice with 50 ml. of ether. The ether extracts were combined with the toluene, washed with a solution of  $\text{Na}_2\text{CO}_3$ , dried over  $\text{Na}_2\text{SO}_4$ , and evaporated to dryness under reduced pressure. The residue, 259 mg., was heated 5 minutes in a boiling water bath with 1 gm. of Girard's Reagent T and 2 ml. of glacial acetic acid. After cooling in an ice bath, the mixture was dissolved by shaking with 40 ml. of ice water, 6 ml. of 5 N NaOH, and 25 ml. of chloroform. Pieces of ice were added to keep the mixture cold. The chloroform was separated and the aqueous phase was extracted twice more with 25 ml. of chloroform. The chloroform extract was washed once with 15 ml. of water which was combined with the aqueous residue.

*Non-Ketonic Fraction, Pregnane-3( $\alpha$ ),20( $\alpha$ )-diol*—The chloroform extract was washed with a solution of  $\text{Na}_2\text{CO}_3$ , dried over  $\text{Na}_2\text{SO}_4$ , and distilled. The residue, 100 mg., was dissolved in methanol. After filtration the solution was concentrated to about 1 ml., filtered from an amorphous precipitate, and further concentrated. The few crystals (3 mg.) which separated melted at 234–236°. Removal of methanol and treatment of the residue with acetone gave more crystalline material (15 mg.) which melted at 237–238° after recrystallization from methanol and appeared to have the properties of pregnane-3( $\alpha$ ),20( $\alpha$ )-diol. The crystals were acetylated with pyridine and acetic anhydride. The acetate, after crystallization from methanol, melted at 160–162°. Admixture with authentic pregnane-3( $\alpha$ ),20( $\alpha$ )-diol diacetate (m.p. 160–162°) did not depress the melting point. Chromatographic analysis of the non-ketonic fraction of a duplicate experiment gave only pregnanediol. There was no evidence of the presence of pregnane-3( $\alpha$ ),17,20-triol.

*Ketonic Fraction, Pregnane-3( $\alpha$ ),17-diol-20-one*—The aqueous residue

containing the Girard complex of the ketones was acidified with 7 ml. of concentrated  $\text{H}_2\text{SO}_4$  previously diluted with ice water and allowed to stand overnight with 25 ml. of chloroform. The aqueous phase was extracted twice more with chloroform. The combined extracts were washed with a solution of  $\text{Na}_2\text{CO}_3$ , dried, and distilled. The residue of ketonic fraction weighed 168 mg. On crystallization from methanol, crystals were obtained which melted at  $205\text{--}208^\circ$ . Recrystallization from acetone gave two crops which melted at  $212\text{--}213^\circ$ . These crops were combined and recrystallized from acetone to give 40 mg. of material which melted at  $212\text{--}213^\circ$ ;  $[\alpha]_D^{27} = +51.8^\circ \pm 0.8^\circ$  ( $c = 1.33$  in alcohol). The substance was dried at  $100^\circ$  *in vacuo* for analysis.

*Analysis*— $\text{C}_{21}\text{H}_{34}\text{O}_3$ . Calculated, C 75.40, H 10.25; found, C 75.22, H 10.18

Other crops weighed 16 mg. and melted at  $210\text{--}213^\circ$ . The remainder (25 mg.) in the mother liquors was dissolved in carbon tetrachloride and chromatographed on 5 gm. of magnesium silicate-Celite (3:5). On elution with various mixtures of carbon tetrachloride and benzene, benzene, and benzene containing 0.2 per cent of alcohol there was no yield from the column. Sixteen 25 ml. portions of benzene containing 0.5 per cent of alcohol were collected. Fractions 4 through 12 melted at  $205^\circ$  or higher and weighed 19 mg.

In another experiment the ketonic fraction was submitted to chromatographic analysis on alumina. The crystalline fractions so obtained appeared to be mixtures which could not be resolved with the amounts available. In view of the experience of Hirschmann and Hirschmann (4) with  $\Delta^5$ -pregnene-3( $\beta$ ), 17-diol-20-one it seems probable that contact with the alumina resulted in considerable conversion to the D-homo compound.

*Pregnane-3( $\alpha$ ), 17-diol-20-one Acetate*—A portion (15 mg.) of the sample that melted at  $212\text{--}213^\circ$  was heated 30 minutes at  $90^\circ$  with 8 drops of pyridine and 4 drops of acetic anhydride. After cooling, water and  $\text{HCl}$  were added. The acetate was filtered out and crystallized from acetone and then from methanol. It melted at  $196\text{--}198^\circ$ . Lieberman and Dobriner (3) gave a melting point of  $201\text{--}202^\circ$ , but we were unable to raise the melting point of our acetate.

We are indebted to Dr. K. Hofmann of the University of Pittsburgh for preparation of the acetate during the earlier part of this investigation. Two of his samples melted at  $195\text{--}198^\circ$  and  $195\text{--}197^\circ$ , respectively. Mixtures of these specimens and the one just described (m.p.  $196\text{--}198^\circ$ ) melted at  $196\text{--}198^\circ$ . One of Dr. Hofmann's samples was sublimed at  $170^\circ$  and 0.005 mm. for analysis.

*Analysis*— $\text{C}_{23}\text{H}_{36}\text{O}_4$ . Calculated. C 73.36, H 9.63  
Found. " 73.51, " 10.13  
" " 73.43, " 10.26

*Oxidation of Pregnane-3( $\alpha$ ),17-diol-20-one Acetate*—The 19 mg. of ketone obtained from the column were acetylated with acetic anhydride and pyridine. Crystallization gave 13 mg. (0.04 mm) of acetate which melted at 198°. It was dissolved in 0.5 ml. of glacial acetic acid and treated with 0.13 ml. of a 0.645 N solution of chromic acid in acetic acid. After standing overnight, benzene and water were added and the aqueous phase was extracted twice more with benzene. The extract was washed with sodium carbonate solution, dried over  $\text{Na}_2\text{SO}_4$ , and evaporated. The residue gave crystals from methanol which melted at 172–180° and which appeared to be unchanged starting material. The methanol was removed and the residue was extracted with hot petroleum ether (b.p. 30–60°). On concentration of the petroleum ether solution to a small volume a small amount of material melting at 158–170° was obtained. The petroleum ether was then removed and the residue, dissolved in 1 ml. of methanol, a few drops of 5 N NaOH, and 1 ml. of water, was heated at 90° for 30 minutes. On cooling, an oil separated. It was induced to crystallize by scratching the tube and was recrystallized from dilute acetone to give long needles which showed a transition point at 137° and melted at 148–150°. A mixture with etiocholan-3( $\alpha$ )-ol-17-one (m.p. 148–150°) also melted at 148–150°. The remainder was benzoylated with benzoyl chloride and pyridine. After two crystallizations from dilute methanol the benzoate melted at 160–162° and a mixture with etiocholan-3( $\alpha$ )-ol-17-one benzoate (m.p. 163°) melted at 160–162°.

*Enzymic Hydrolysis of Glucuronide*—A comparison of acid and enzymic hydrolysis was made. The acid hydrolysis of 76 mg. of purified glucuronide was carried out essentially as previously described (1). Enzymic hydrolysis of 76 mg., according to the conditions of Mason and Kepler (7), was carried out with rat liver enzyme prepared by the method of Talbot, Ryan, and Wolfe (11). Acid hydrolysis gave 41 mg. of neutral fraction, 38 mg. of which were separated with the aid of Girard's Reagent T into 8 mg. of non-ketonic fraction and 25 mg. of ketonic fraction. The corresponding fractions obtained by enzymic hydrolysis were 43, 12, and 24 mg. A blank run on the enzyme preparation gave 3.5 mg. of neutral fraction.

The ketonic fractions were crystallized from acetone. The first crops melted at 212–213° (acid hydrolysis) and 213–214° (enzymic hydrolysis), respectively. Additional crops gave total yields of material of 10.5 and 12.7 mg., respectively, which melted above 210°.

Since the ketone obtained by enzymic hydrolysis had been subjected to a milder procedure than that obtained by acid hydrolysis, its specific rotation was determined:  $[\alpha]_D^{28} = +52.4^\circ \pm 2.4^\circ$  ( $c = 0.420$  in 95 per cent alcohol). This value is the same, within the limits of experimental error, as that determined for the ketone previously obtained by acid hydrolysis.

These results do not show any essential differences in properties or in yields between the preparations obtained by acid and enzymic hydrolysis.

Dr. C. B. Shaffer, The Mellon Institute, and Evelyn W. Strickler had a part in this investigation. Our thanks are due them for their contributions. Thanks are also due Martin Kalser, Elsa DeBona, and Henry Karpinski who helped process the urine.

Analyses were made by Wm. Saschek, A. J. Haagen-Smit, and J. F. Alicino.

#### SUMMARY

A glucuronide was isolated as the sodium salt, having the composition  $C_{27}H_{41}O_9Na$ , from the urine of a feminine pseudohermaphrodite. Pregnane-3( $\alpha$ ), 17-diol-20-one was obtained from the glucuronide by hydrolysis and its structure proved by degradation to be etiocholan-3( $\alpha$ )-ol-17-one. A crude preparation of the sodium glucuronidate also contained pregnane-diol, but pregnane-3( $\alpha$ ), 17, 20-triol was not found.

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# FACTORS WHICH INFLUENCE THE STABILITY OF TRYPTOPHAN DURING THE HYDROLYSIS OF PROTEINS IN ALKALINE SOLUTION\*

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The stability of tryptophan, when heated in alkaline solution under conditions suitable for the hydrolysis of proteins, has been studied by a number of investigators. In general, the older reports indicate that a somewhat irregular loss of tryptophan usually occurs. With regard to the causes of this loss, the report of Lugg (1), showing that the addition of stannite resulted in partial stabilization of tryptophan, is of particular significance as it indicates that the destructive reaction is one of oxidation. The results of Brand and Kassell (2) are in agreement with this conception. The production of indole from proteins as well as from pure tryptophan by heating these substances in alkaline solutions was studied by Herzfeld (3). When relatively large amounts of copper sulfate were added, 60 per cent of the tryptophan was converted to indole. This suggests that even traces of copper or other heavy metals might influence the stability of tryptophan.

The wide-spread adoption of microbiological methods for amino acid analysis focuses new attention on the best method for the hydrolysis of proteins and especially of foodstuffs for tryptophan determination. Greene and Black (4) found autoclaving with 5 N Ba(OH)<sub>2</sub> for 5 hours a satisfactory procedure. Stokes *et al.* (5) adopted the procedure of autoclaving for 10 hours with 5 N NaOH. Hauschildt *et al.* (6) also used sodium hydroxide for hydrolysis and apparently considered it satisfactory. In contrast with these reports, Wooley and Sebrell (7) found tryptophan to be relatively unstable when autoclaved with NaOH. The report of Greenhut *et al.* (8) indicated the same thing.

These differences in results presumably were due to differences in material or conditions. It is the purpose of this report to present data showing some of the factors which can alter the stability of tryptophan and to present a procedure by which tryptophan can be completely stabilized during the

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hydrolysis of proteins and foodstuffs with sodium hydroxide. This procedure has the advantage that nothing is added which must be removed prior to the determination of tryptophan by microbiological methods.

A method for the determination of tryptophan with *Streptococcus faecalis* R is described. This organism was selected on the basis of its specificity. Indole and anthranilic acid, which have considerable tryptophan activity for *Lactobacillus arabinosus* (9, 10), are completely inactive for *Streptococcus faecalis* R (9, 11).

#### EXPERIMENTAL

*Determination of Tryptophan with Streptococcus faecalis R*—The medium is given in Table I. The composition of the salt solutions has been pre-

TABLE I  
*Medium for Determination of Tryptophan with Streptococcus faecalis R\**

H <sub>2</sub> O <sub>2</sub> -treated peptone solution (50 mg. per ml.)	200 ml.	DL-Methionine	200 mg.
Glucose	40 gm.	L-Cystine	200 "
Succinic acid	20 "	L-Tyrosine	200 "
Sodium acetate	6 "	Thiamine chloride	0.4 "
Adenine sulfate	10 mg.	Pyridoxamine	0.8 "
Guanine	10 "	Calcium pantothenate	0.4 "
Uracil	10 "	Riboflavin	1.0 "
Xanthine	10 "	Niacin	2.0 "
Salts 1	10 ml.	Biotin	2 "
" 2	10 "	p-Aminobenzoic acid	2 "
" 3	10 "	Folic acid (synthetic)	8 "

Dilute to approximately 800 ml.; add 12 gm. NaOH pellets and finish neutralizing with NaOH solution; dilute to final volume of 1 liter

\* Medium for 200 cultures of 10 ml. final volume (5 ml. of above medium per culture).

viously reported (12). Hydrogen peroxide-treated peptone is prepared as follows: 50 gm. of Bacto-peptone are dissolved in 250 ml. of water, and 250 ml. of 1 N hydrochloric acid are added after the peptone is completely dissolved. 0.02 mole of hydrogen peroxide (2.3 gm. of 30 per cent H<sub>2</sub>O<sub>2</sub>) is added and the solution is allowed to stand overnight at room temperature. The material is then steamed 30 minutes at atmospheric pressure, stirred while hot, cooled, neutralized with sodium hydroxide, and steamed for 1 hour. The solution is ready for use after diluting to a final volume of 1 liter. A reagent grade of hydrogen peroxide which does not contain any preservative should be used. The amount of hydrogen peroxide indicated above is less than was used in the preliminary report (13). This modification results in an improved medium for the determination of tryptophan.

The assay procedure and the technique of handling the bacteria are the

same as that previously used for the determination of histidine with *Streptococcus faecalis* R (12).

In accordance with the reports of Stokes *et al.* (5) and Baumgarten *et al.* (11) it was found that DL-tryptophan has exactly one-half the activity of L-tryptophan. Complete racemization of tryptophan in hydrolysates prepared with base is assumed. Therefore direct analytical results are obtained with a DL standard. If L-tryptophan is used as a standard, the analytical values should be multiplied by 2. The range of the standard curve is from 0 to 22  $\gamma$  of DL-tryptophan. The maximum titration value is approximately 4 ml. of 0.1 N sodium hydroxide (5 ml. aliquot from a 10 ml.

TABLE II

*Stability of Tryptophan Autoclaved in 4 N Sodium Hydroxide with Various Substances*

DL-Tryptophan added	Other material added*	Time of autoclaving	Tryptophan found	Recovery
mg.		hrs.	mg.	per cent
0.625		8	0.514, 0.522	82.9
1.25		8	1.09, 1.09	87.3
2.5		8	2.36, 2.38	94.8
5.0		8	4.70, 4.72	94.2
2.5		16	2.23, 2.25	89.6
2.5	CuSO <sub>4</sub> ·5H <sub>2</sub> O	8	2.16, 2.12	85.6
2.5	FeCl <sub>3</sub> ·6H <sub>2</sub> O	8	2.27	90.8
2.5	NiCl <sub>2</sub> ·6H <sub>2</sub> O	8	2.31	92.1
2.5	SnCl <sub>2</sub> ·2H <sub>2</sub> O	8	2.34, 2.41	95.2
0.625	Cysteine	8	0.620, 0.623	99.6
2.5	"	8	2.55, 2.52	101.4
2.5	"	16	2.54, 2.52	101.2
2.5	"	30	2.49, 2.50	99.8
2.5	" + CuSO <sub>4</sub> ·5H <sub>2</sub> O	8	2.52, 2.51	100.6
2.5	" + NiCl <sub>2</sub> ·6H <sub>2</sub> O	8	2.48, 2.56	100.2
2.5	" + SnCl <sub>2</sub> ·2H <sub>2</sub> O	8	2.51, 2.52	101.2
2.5	" + FeCl <sub>3</sub> ·6H <sub>2</sub> O	8	2.54	101.8

\* 0.4 mg. of each of the metal salts indicated was added.

culture). Attention must be given to the purity of the standard. The majority of commercial tryptophan samples are not pure enough for this purpose.

*Preparation of Hydrolysates by Enzymatic Digestion*—Enzymatic hydrolysates were prepared according to the method of Greenhut, Schweigert, and Elvehjem (8). Commercial enzyme preparations were used.

## RESULTS AND DISCUSSION

Preliminary tests resulted in the following findings: When DL-tryptophan was autoclaved in 3 or 4 N sodium hydroxide for various lengths of time,

TABLE III

*Effect on Stability of Tryptophan of Autoclaving in Sealed Tubes*

Material autoclaved	Concentration of NaOH	Other conditions	DL-Tryptophan found	Recovery
			mg.	per cent
DL-Tryptophan, 1.25 mg.	4 N	Sealed in air	0.46	36.9
“ 1.25 “	4 “	“ “ “	0.44	35.5
“ 1.25 “	5 “	“ “ “	0.43	34.3
“ 1.25 “	5 “	“ “ “	0.42	33.7
“ 1.25 “	4 “	Cysteine added, air displaced by N <sub>2</sub>	1.24	99.2
“ 1.25 “	5 “	“ “	1.28	102.3
“ 1.25 “	5 “	“ “	1.25	100.0
Casein, 250 mg.	4 “	Sealed in air	2.80	
“ 250 “	4 “	“ “ “	2.78	
“ 250 “	4 “	Cysteine added, air displaced by N <sub>2</sub>	2.99	
“ 250 “	4 “	“ “	3.01	

TABLE IV

*Liberation of Tryptophan from Casein and Beef Fibrin under Various Conditions of Hydrolysis*

Hydrolyzing agent	Time of hydrolysis	Tryptophan in casein	Tryptophan in beef fibrin
	hrs.	per cent	per cent
3 N NaOH with cysteine . . . . .	8	1.10	2.77
3 “ “ “ “ . . . . .	16	1.13	2.95
4 “ “ “ “ . . . . .	2	1.00	2.76
4 “ “ “ “ . . . . .	4	1.09	2.79
4 “ “ “ “ . . . . .	6	1.11	2.85
4 “ “ “ “ . . . . .	8	1.12	3.01
4 “ “ “ “ . . . . .	12	1.15	3.09
4 “ “ “ “ . . . . .	16	1.17	3.17
5 “ “ “ “ . . . . .	4	1.17	
5 “ “ “ “ . . . . .	8	1.12	2.84
5 “ “ “ “ . . . . .	16	1.20	
3 “ Ba(OH) <sub>2</sub> . . . . .	8		3.23
3 “ “ . . . . .	12	1.15	
6 “ “ . . . . .	4	1.24	
6 “ “ . . . . .	8	1.24	3.42
6 “ “ . . . . .	16	1.21	3.38
Pancreatin + erepsin . . . . .	24	1.07	2.92

a part of the tryptophan was always destroyed. The extent of destruction was variable and appeared to have no consistent relationship to the length of time of autoclaving. Mixtures of pure amino acids patterned after

typical protein hydrolysates afforded partial protection. On testing the amino acids separately, it was found that the protective action was due to cystine and methionine. Cysteine proved to be a more effective protective agent than either of these two amino acids.

Some stability tests of particular significance are given in Table II. These data show that (1) in the absence of a protective agent the percentage loss of tryptophan was related to the total concentration of tryptophan, the greater percentage loss occurring at the lower concentrations, (2) that the loss of tryptophan was greatly enhanced by the addition of copper

TABLE V  
*Tryptophan Content of Some Foodstuffs*

Material analyzed	Protein content	Tryptophan found in protein*		
		Autoclaved 16 hrs., 4 N NaOH	Autoclaved 8 hrs., 6 N Ba(OH) <sub>2</sub>	Digested with pancreatin + crepsin
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Beef tongue†	81.45	1.25	1.10	0.96
“ “	83.10	1.19	1.07	0.96
“ loin†	82.60	1.27	1.18	1.11
“ “	87.50	1.24	1.11	1.07
“ liver†	68.85	1.46	1.41	1.36
“ “	69.30	1.52	1.47	1.42
Cottonseed meal	43.19	1.43	1.24	
“ “	40.26	1.50	1.35	
Peanut meal	38.69	1.11	0.92	
Soy bean meal	43.32	1.64	1.45	
Dried skim milk	33.44	1.42	1.29	
Tankage	58.63	1.00	0.91	
Meat scraps	52.17	0.69	0.69	
Alfalfa leaf meal	21.32	1.63	1.40	

\* This is equivalent to calculating to 16 per cent nitrogen.

† Moisture-free-fat-free samples.

and was somewhat influenced by iron and nickel, and that (3) by the use of cysteine, the destruction of tryptophan was completely prevented even in the presence of heavy metals. These findings are in agreement with the hypothesis that the loss of tryptophan is a result of oxidation catalyzed by heavy metals.

To test this hypothesis further, tryptophan solutions were autoclaved in sealed tubes containing air or nitrogen. Cysteine was also added to the tubes containing nitrogen. The ratio of air space to liquid volume was about 1:1 and the tubes containing air were tilted to increase the surface

area. Nearly two-thirds of the tryptophan in the tubes containing air was destroyed, while complete recoveries were obtained under nitrogen (Table III). With casein, this effect was much less pronounced, presumably because of the protective action of the sulfur-containing amino acids present in the protein.

The procedure which resulted in the complete protection of tryptophan in the stability tests is given below. This procedure was also used to prepare hydrolysates of proteins and foodstuffs for tryptophan analysis.

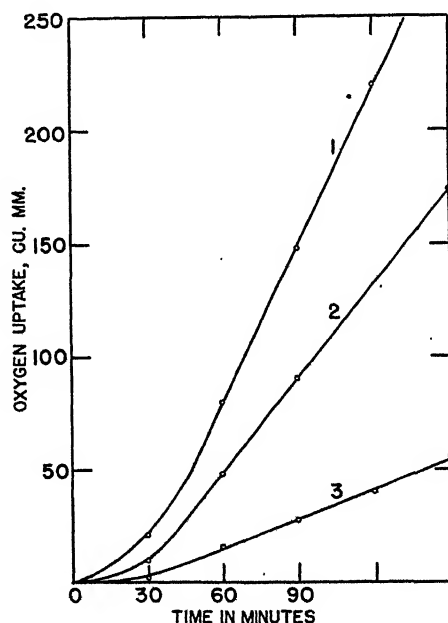


FIG. 1. Effect of copper concentration on oxidation of tryptophan with molecular oxygen. 0.01 mm of DL-tryptophan in 2.5 ml. of 4 N sodium hydroxide, oxygen atmosphere; 38°. Curve 1, 0.002 mm of copper; Curve 2, 0.001 mm of copper, Curve 3, 0.005 mm of copper.

100 mg. of L-cysteine hydrochloride were added to 16 ml. of 4 N sodium hydroxide in a 100 ml. Pyrex beaker. Another beaker of larger size was used as a cover. The solution was autoclaved at 15 pounds pressure for about 1 hour. While hot, another 100 mg. portion of cysteine was added along with the tryptophan or 0.5 gm. of sample to be hydrolyzed. Autoclaving was then continued for the time indicated.

Hydrolysates prepared in this manner were analyzed for tryptophan and the results compared with those obtained after hydrolysis with barium

hydroxide and with digestive enzymes. From Table IV it will be seen that tryptophan is more readily liberated from casein than from fibrin. The maximum value for tryptophan in each of these proteins was obtained after treatment with 6 N  $\text{Ba}(\text{OH})_2$  for 8 hours. The enzymatic technique of Greenhut *et al.* (8) gave slightly lower results.

The tryptophan content of some foodstuffs is indicated in Table V. It will be noted that, in general, hydrolysis with sodium hydroxide gave higher values than hydrolysis with barium hydroxide.

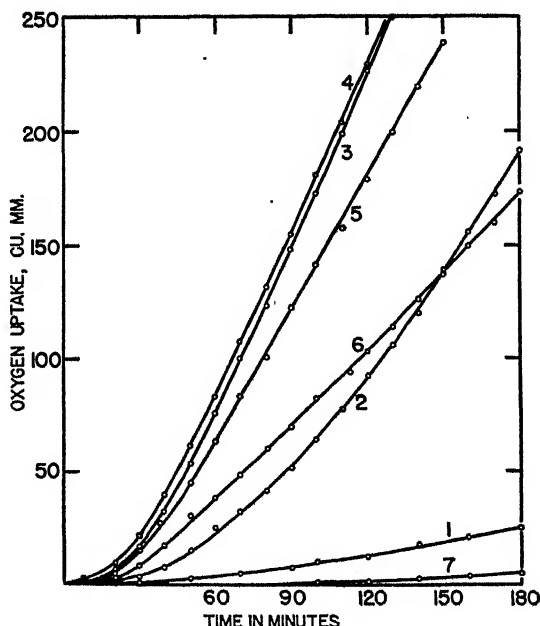


FIG. 2. Effect of sodium hydroxide concentration on oxidation of tryptophan. 0.01 mm of DL-tryptophan in 2.5 ml., oxygen atmosphere, 38°, 0.002 mm of copper. Curves 1 to 6 designate sodium hydroxide normality; Curve 7, phosphate buffer pH 7.4.

Manometric tests with the Barcroft-Warburg apparatus showed that the reaction of tryptophan with molecular oxygen takes place even at room temperature. The reaction was catalyzed by copper (Fig. 1) and influenced by the concentration of sodium hydroxide (Fig. 2). It is to be expected that at elevated temperatures much smaller quantities of copper would be effective. Under the conditions of these experiments, the maximum rate of oxygen consumption took place in 4 N sodium hydroxide. The decreased rate of reaction with higher concentrations of base was attributed to lower

solubility of oxygen. The reaction proceeded at a measurable rate even in phosphate buffer at pH 7.4. Some catalytic effect was observed with nickel and platinum.

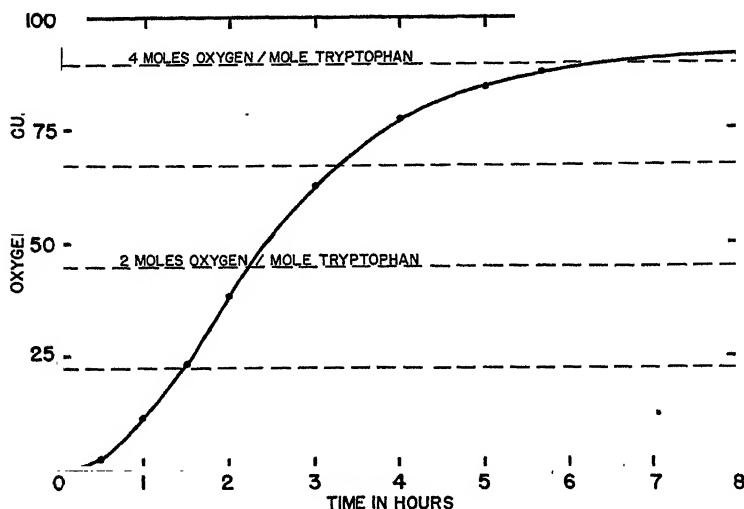


FIG. 3. Oxidation of tryptophan with molecular oxygen and copper catalyst. 0.001 mm of DL-tryptophan in 2.5 ml. of 4 N sodium hydroxide, 0.002 mm of copper, 38°, oxygen atmosphere.

TABLE VI

*Relative Microbiological Activity of Tryptophan Oxidized with Molecular Oxygen*

Atoms of oxygen consumed per mole of tryptophan	Activity for <i>Lactobacillus arabinosus</i> 17-5	Activity for <i>Streptococcus faecalis</i> R
0	100	100
1	84	80
2	63	59
3	48	42
4	37	29
5	27	18
6	16	5
7	16	2
8	17	0
10	13	

A short induction period was typical of the manometric experiments. This was followed by rapid consumption of 4 moles of oxygen (Fig. 3). The reaction continued very slowly beyond this point with the consumption

of several more moles of oxygen. It was of interest to stop the reaction at various stages to test the biological activity of the partially oxidized material. Table VI shows that *Lactobacillus arabinosus* 17-5 had greater ability to utilize the oxidized compounds than did *Streptococcus faecalis* R. With the consumption of 4 moles of oxygen, all of the activity for *Streptococcus faecalis* R was lost. In the experiments by Herzfeld (3) in which small amounts of tryptophan were heated with relatively large amounts of copper sulfate, in alkaline solution, a 60 per cent yield of indole was obtained. If indole were the major end-product in the experiments reported here, greater activity for *Lactobacillus arabinosus* would be expected. Color

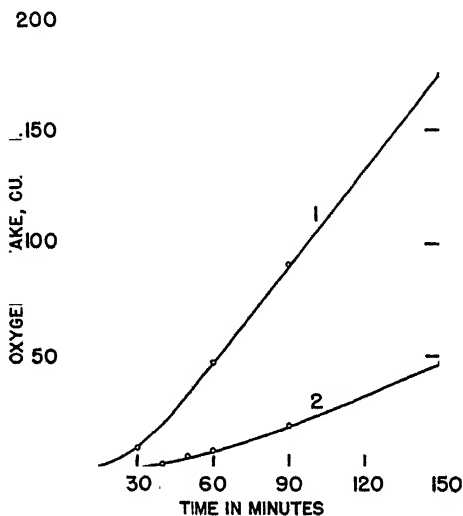


FIG. 4. Effect of methionine on oxidation of tryptophan with copper as catalyst. 0.01 mm of DL-tryptophan, 0.001 mm of copper. Curve 1, control without methionine; Curve 2, 0.1 mm of DL-methionine.

tests (14) on the solutions at various stages of oxidation did not show the presence of indole.

Cysteine reacts very rapidly with oxygen in alkaline solution. This indicates that at least one of the mechanisms by which cysteine stabilizes tryptophan is by removing oxygen from solution. Cysteine or decomposition products therefrom might also function by combining with traces of copper or other heavy metals so that the catalytic action is blocked. Evidence for this mechanism was found in the case of methionine. This amino acid was not oxidized under the experimental conditions but did partially stabilize tryptophan (Fig. 4).



## SUMMARY

1. Evidence is presented which indicates that the destruction of tryptophan which occurs during autoclaving in sodium hydroxide solution is due to oxidation by molecular oxygen.

2. A procedure is described by which this destruction can be completely prevented. Cysteine is used as the stabilizing agent. This procedure was utilized for the preparation of hydrolysates of various foodstuffs for tryptophan analysis.

3. Tests with the Barcroft-Warburg apparatus showed that tryptophan reacts with molecular oxygen in alkaline solution at room temperature. Copper salts greatly accelerate the rate of this reaction. Some catalytic effect was also observed with nickel and platinum.

4. A simplified medium for the determination of tryptophan with *Streptococcus faecalis* R is described. This medium utilizes hydrogen peroxide-treated peptone as a source of most of the amino acids.

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# THE TRYPTOPHAN CONTENT OF MEAT\*

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It is the purpose of this communication to report the tryptophan content of various kinds of meat as determined by a microbiological method with *Streptococcus faecalis* R as the test organism. Representative samples were also analyzed by a chemical procedure.

Meat samples which had been dehydrated from the frozen state and then defatted were used in this investigation. These samples were prepared in order that as many of the amino acids as possible might be determined on the same materials. Methionine (1) and histidine (2) studies have been previously reported.

## EXPERIMENTAL

Description of the samples and details of their preparation have been previously given (1). The equivalence of the dried solids in terms of fresh meat was known from nitrogen determinations on the fresh and dehydrated materials. In all cases in which the extracted fat contained as much as 1 per cent of the total nitrogen, this was taken into consideration in calculating the equivalence values.

The samples were hydrolyzed for microbiological assay in the autoclave with 4 N sodium hydroxide for 16 hours. Cysteine was used as a stabilizing agent according to the procedure described in the preceding publication (3). Tryptophan was determined microbiologically by the method of Kuiken, Lyman, and Hale (3). Chemical determinations were made by the method of Graham, Smith, Hier, and Klein (4).

## RESULTS AND DISCUSSION

The protein and tryptophan content of the fresh meat and also the average tryptophan content of the meat proteins are given in Table I. In most cases, variations in the tryptophan content of the three samples of the same kind of tissue (all from different animals) were quite small. The agreement was even better when the data were expressed as percentage of

\* This investigation was supported by a grant from the American Meat Institute.

TABLE  
Tryptophan Content.

Tissue	Tryptophan in fresh tissues						Tryptophan in protein*		Bibliographic reference No.
	Sample 1		Sample 2		Sample 3		Average tryptophan content	Literature values	
	Protein	Tryptophan	Protein	Tryptophan	Protein	Tryptophan			
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	
Beef loin.....	20.20	0.26	22.65	0.28	22.10	0.27	0.27	1.26,† 1.35, ‡	(4, 5)
“ brisket.....	18.85	0.23	21.28	0.26	21.32	0.26	0.25	1.35, 1.3,	(6, 7)
“ round.....	22.05	0.27	19.88	0.24			0.26	1.17, 1.3, 1.24	(8, 9, 10)
Pork loin.....	20.14	0.26	20.67	0.27	20.78	0.25	0.26	1.31, 1.31, 1.28§	(5, 6, 11)
Lamb chop.....	18.85	0.24	20.91	0.25	20.12	0.25	0.25	1.44, 1.44	(5, 6)
Beef liver.....	17.94	0.26	18.56	0.28	18.69	0.31	0.28	1.81, 1.55, 1.60, 1.2, 1.38	(5, 6, 11) (7, 12)
Pork “ .....	19.91	0.34	20.44	0.35	18.09	0.27	0.32	1.63	
Lamb “ .....	22.25	0.36	21.56	0.36	21.00	0.36	0.36	1.67	
Beef tongue.....	17.65	0.22	16.15	0.20	17.60	0.21	0.21	1.22	(11)
Pork “ .....	16.31	0.21	16.25	0.22	15.18	0.20	0.21	1.31	
Beef heart.....	17.35	0.24	18.20	0.24	17.28	0.24	0.24	1.36	(5, 7, 11)
Pork “ .....	17.59	0.25	16.34	0.22	16.88	0.24	0.24	1.43	
Lamb “ .....	16.62	0.22	16.41	0.22	16.12	0.22	0.22	1.37	
Beef kidney.....	16.95	0.25	18.22	0.29	18.10	0.26	0.27	1.49	(11, 7)
Pork “ .....	15.72	0.25	15.31	0.25	15.56	0.25	0.25	1.60	
Lamb “ .....	14.72	0.21	15.56	0.24	15.54	9.23	0.23	1.54	
Beef brain.....	10.75	0.14	10.65	0.14	10.55	0.15	0.14	1.35	(5, 7)
“ thymus.....	15.90	0.14	16.40	0.13	15.50	0.13	0.13	0.83	(7)
“ spleen.....	18.30	0.22	18.35	0.23	18.60	0.23	0.23	1.21	(7)

\* Calculated to 16 per cent nitrogen.

† 16.2 per cent nitrogen.

‡ Beef muscle tissue.

§ Average of three values reported.

the protein. Liver was the exception. The values for the tryptophan content of the beef liver samples were verified by repeated analyses.

Significant differences were found in the tryptophan content of the protein in the various organs, but the tryptophan content of the same organ from beef, pork, and lamb was very nearly the same. As compared with muscle tissue, the tryptophan content of liver and kidney was substantially higher. Thymus protein contained much less tryptophan than any other tissue analyzed.

Agreement between the values found in the literature for the tryptophan content of different kinds of meat is better than for some of the other amino acids. The value recently reported by Graham and associates (4) for beef muscle and the values given by Greenhut, Schweigert, and Elvehjem (11) for beef liver, tongue, heart, and kidney are in excellent agreement with the data presented here. Beach, Munks, and Robinson (5) determined tryptophan

TABLE II  
*Comparative Data Obtained by Chemical and Microbiological Methods*

Tissue	Tryptophan in protein*	
	Microbiological assay	Chemical analysis
	<i>per cent</i>	<i>per cent</i>
Pork liver . . . . .	1.71	1.65
Beef " 1 . . . . .	1.52	1.59
" " 2 . . . . .	1.66	1.61
" loin 1 . . . . .	1.28	1.33
" " 2 . . . . .	1.21	1.29
" brisket . . . . .	1.21	1.27

\* Calculated to 16 per cent nitrogen.

tophan on meat samples which had been washed with boiling water. It is to be expected that the loss of non-protein nitrogen by this procedure would result in slightly higher tryptophan values when the data are expressed as percentage of the protein (calculated to 16 per cent nitrogen). In view of this difference in the method of preparing the samples, it was considered that the data presented here are in very satisfactory agreement with the report of these investigators.

It was of interest to compare data obtained by a chemical method of analysis and by microbiological assay of identical samples. Table II shows that the values obtained by the two methods were in satisfactory agreement.

#### SUMMARY

The tryptophan content of 56 samples of meat was determined by a microbiological assay procedure. The test organism was *Streptococcus faecalis* R.

The samples of meat included beef loin, round, brisket, liver, heart, kidney, tongue, brain, thymus (sweetbreads), and spleen, as well as pork loin, liver, heart, kidney, and tongue, and lamb chops, liver, kidney, and heart.

In most cases, variations in the tryptophan content of the same kind of meat taken from different animals were quite small. As compared to muscle tissues, the tryptophan content of liver and kidney was substantially higher. Thymus protein contained considerably less tryptophan than any other tissue analyzed. For a given type of tissue, the tryptophan content was very nearly the same whether it came from beef, pork, or lamb.

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# IDENTIFICATION OF THE TRYPSIN INHIBITOR OF EGG WHITE WITH OVOMUCOID\*

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The trypsin inhibiting action of egg white, which has been known for over 40 years, has not been identified with any of the recognized components (1) of egg white. Suggestions made in this regard include the postulates (a) that difficultly digestible proteins of egg white displaced trypsin from the substrate (casein) (2), (b) that the antitrypsin is associated with the globulin fraction of egg white (3), and (c) that the inhibitor is probably a protein hydrolysis product but not a true protein (4). Balls and Swenson (4), who made the most complete study of the inhibitor, found that it was neither lipid nor carbohydrate (exclusively) and that it had about the same nitrogen content (slightly low), optical rotation, and sulfur content as are now recognized for ovomucoid. Meyer *et al.* (5) reported that a highly active antitrypsin sample sent them by Dr. Swenson showed the properties and composition of an egg mucoid. They stated that "the inhibitory effect of egg white preparations on tryptic activity is probably due to a mucoid." However, the activity reported by Balls and Swenson for their best preparation indicated that the inhibitor represented less than 1 per cent of the egg white solids, which, of course, is less than one-tenth of the ovomucoid in egg white. Possible explanation of this result, which conflicts with the results reported in this paper, may lie in the use for assay purposes of enzyme precursors, which were activated by enterokinase, generally in the presence of inhibitor. The complication introduced by the use of precursors and enterokinase (a common practice at the time the work was done) is illustrated by the reports that the inhibitor was an antikinase (6), that it possibly acted by displacing enterokinase from its combination with the enzyme (4), and that the inhibitor was not an antikinase (7, 2).

This report concerns a component of egg white that inhibits trypsin containing no enterokinase. Primary consideration is given to data indicating that antitryptic activity is a characteristic of "native" ovomucoid. The considerations include the distribution of the inhibitor in hen's eggs, preparation and fractional precipitation of the inhibitor, comparison of the

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inhibitor with ovomucoid of the literature, determination of its heat lability, and its stoichiometric relation to trypsin at the 50 per cent inhibition level.

### Methods

*Determination of Antitryptic Activity*—Inhibitory potency was determined by comparing the inhibition of crystallized trypsin caused by a preparation of unknown inhibitor content with the inhibition caused by a sample of egg white, which was used as a standard throughout the investigation. Generally the trypsin method of Anson (8) was used; however, a casein formol titration procedure gave identical results. With the Anson

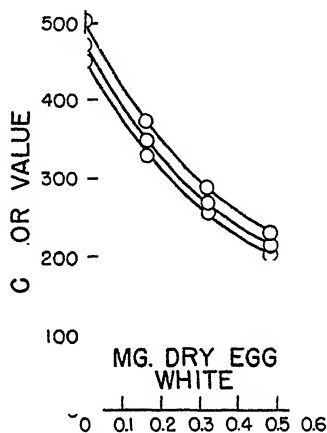


FIG. 1. Standard curves relating color value (tryptic activity) and amount of dry egg white in reaction mixture at three slightly differing trypsin concentrations.

method, suitable amounts of inhibitor were added to 5 ml. of hemoglobin substrate followed by a standard amount of trypsin. Otherwise the usual trypsin assay procedure was followed exactly. 1 unit of inhibitor is defined as the amount of inhibitor contained in 1 mg. of standard dried egg white. The relation between color value and mg. of a "standard" egg white (dry weight basis) is shown in Fig. 1. Such standard curves were used to determine antitrypsin potency. The several parameters were obtained to facilitate interpolations necessitated by small variations in the color obtained in the control run without inhibitor. This resulted from slow loss of activity of trypsin at 5° in aqueous solution. Fresh trypsin solution was prepared at least biweekly. All samples of egg white tested have had the same inhibitor potency within experimental error ( $\pm 10$  per cent). Samples of egg white used as standards contained about 15 per cent moisture; however, the activities were related to total solids in all cases.

*Total nitrogen* was determined by the micro-Kjeldahl method with HgO as a catalyst. *Amino nitrogen* was determined by the manometric Van Slyke method with a 15 minute reaction time. *Optical rotation* was determined in 5 per cent aqueous solution. *Total sugar* was determined by the cyanide methods described by Militzer (9) on an acid hydrolysate prepared as described below for cystine analysis. *Moisture content* was obtained by determining the loss in weight at 70° in a vacuum oven. *The molecular weight* was determined by the osmotic pressure method of Bull (10). A 1 per cent solution of inhibitor in 0.1 M NaCl was used in these measurements. In order to facilitate the development of equilibrium conditions, fresh membranes were used after three or four determinations. *Acetyl* content was determined by the method described by Elek and Harte (11). *Total sulfur* was determined by the sodium peroxide fusion method. *Tyrosine and tryptophan* analyses were made by the methods of Thomas (12) and of Horn and Jones (13), respectively. *Cystine* was determined by the method of Sullivan (14). Hydrolysis was carried out by refluxing a 0.5 gm. sample with 12 ml. of 18 per cent HCl for 7½ hours in an oil bath at 145° ± 5°. The hydrolysate was mixed with charcoal (norit A), filtered, and washed with 0.1 N HCl. The filtrate was adjusted to pH 3.5, made to 50 ml. with 0.1 N HCl, and analyzed according to the procedure described in the reference.

### Results

*Occurrence of Antitryptic Activity in Various Fractions of Hen's Eggs*—Antitryptic activity was found to occur almost exclusively in egg white. Table I shows that egg yolk contains only about 0.4 per cent as much antitryptic activity as egg white (dry weight basis). The antitryptic activity of livetin calculated to a whole yolk basis ( $0.060 \times 0.067 = 0.0040$  unit) indicates that the yolk trypsin inhibitor appears quantitatively in the livetin fraction of the yolk. The livetin sample used in this experiment was prepared from other eggs than those used in the assay of whole yolk. Studies of the variation in inhibitor content of the yolk and the nature of the yolk inhibitor have not been undertaken. Nevertheless, these data would appear to be the best, though inconclusive, evidence that small amounts of ovomucoid occur in fresh egg yolk. An indication that ovomucoid occurs in fresh egg yolk is obtained from the results of Fraenkel and Jellinek (16), who found that commercial "yolk albumin" from lecithin preparation contained carbohydrate identical with that in ovomucoid. Their results are inconclusive, not only because of the inferential nature of the argument based on the identity of carbohydrate but also because it seems likely that the "yolk albumin" used by them was contaminated with egg white.



The antitryptic activity of egg white was found to be equally distributed between the thin and thick white within experimental error (Table I). The reported values are the average of closely agreeing results for three eggs analyzed separately. The solids content of the thin and thick white averaged 12.8 ( $\pm 0.1$ ) and 13.0 ( $\pm 0.1$ ) per cent, respectively. This equal distribution of antitryptic activity in thick and thin egg white differs from the

TABLE I  
*Inhibitor Activity of Components and Fractions of Hen's Eggs*

Component or fraction of egg	Inhibitor activity (dry weight basis)	Inhibitor, yield*
	<i>units per mg.</i>	<i>per cent</i>
Egg white. (a) Whole (standard) . . . . .	1.00	100
(b) Thin . . . . .	1.05	
(c) Thick . . . . .	1.03	
Egg yolk† . . . . .	0.004	
Livetin . . . . .	0.060	
Ovalbumin. (a) $(\text{NH}_4)_2\text{SO}_4$ ppt. . . . .	1.2	72
(b) 3 times recrystallized . . . . .	<0.2	<12
Conalbumin . . . . .	<0.2	<3
Globulin fraction . . . . .	1.0	12
Lysozyme . . . . .	<0.2	<1
Ovomucoid. (a) Prepared by heating . . . . .	1.1	14
(b) Prepared without heating . . . . .	9.0	105
Avidin. (a) Crude . . . . .	3.0	
(b) Purified . . . . .	<0.2	

\* Calculated on assumptions that fractions tested were pure and that dry egg white contains 60 per cent ovalbumin, 14 per cent conalbumin, 12 per cent globulins, 3 per cent lysozyme, and 11 per cent ovomucoid, unaccounted for 3 per cent. These assumptions are approximations but serve the present purpose. The percentage compositions are based on Table II of Longworth *et al.* (1) and, for ovomucoid, the data of Sørensen (15) (see Table III).

† The yolk and livetin samples were prepared with special care to ensure their purity with respect to egg white. The unbroken yolk was separated from the white, washed in salt solution and then in water, dried by rolling on clean cloth, and finally broken in such a way that the outside of the yolk membrane, which was discarded, practically did not come in contact with the yolk contents.

report of Balls and Swenson (4) that the inhibitor was concentrated in the thin white.

Because of this finding, recognized components of whole egg white, rather than of thin white, were assayed for antitryptic activity. Table I shows that the antitryptic activity of egg white did not appear to be associated with egg albumin (crystalline), conalbumin, the globulins, avidin, or with ovomucoid prepared by a heating procedure. Crude avidin (500 avidin

units per mg.) evidently contains some inhibitor as impurity compared with purified avidin (2500 avidin units per mg.). Ovomucoid prepared without resort to heat treatment possessed high antitryptic activity and on a 100 per cent yield basis contained all of the inhibitor of egg white. It is pertinent that the ovalbumin (Fraction A) obtained by ammonium sulfate precipitation contains about 70 per cent of the antitryptic activity, since Longworth *et al.* (1) showed that this fraction contains considerable ovomucoid. The high antitryptic activity of this fraction is consistent therefore with other results that indicate the identity of the trypsin inhibitor and ovomucoid.

*Preparation of Egg White Trypsin Inhibitor (Ovomucoid)*—Trypsin inhibitor was prepared by the method of Balls and Swenson (4), by salt fractionation, and by three slightly differing procedures involving the use of trichloroacetic acid and acetone. All of these procedures resulted in inhibitor preparations that had the same inhibitor activity, within experimental error. The activity of these preparations indicated about a 9-fold purification of the inhibitor (*cf.* Table I). The trichloroacetic acid-acetone methods were variants of the procedure described below. The Balls and Swenson procedure involved ammoniacal extraction of acetone-ether-dried egg white, heating to 75–80° at pH 5 (acetic acid) for 5 to 10 minutes, and precipitation of the soluble solids with alcohol. The salt fractionation procedure involved removal of globulins at half saturation with sodium sulfate, removal of albumin by crystallization, removal of conalbumin by adjusting the crystallization filtrate to pH 3 in the cold, precipitation of the inhibitor by saturation with ammonium sulfate, solution and dialysis of the inhibitor, and finally, precipitation of the inhibitor with alcohol.

The following trichloroacetic acid-acetone method of preparing the inhibitor is not necessarily the best under mild conditions, but it was satisfactory for our purpose and has been used more or less routinely: Egg white at 25–30° is adjusted to pH 3.5 by the slow addition of about 1 volume of a trichloroacetic acid-acetone solution (1 volume of aqueous 0.5 M trichloroacetic acid plus 2 volumes of acetone). The egg white is stirred thoroughly during the addition and for 15 to 30 minutes thereafter. It should then have a thick granular creamy appearance. The creamy mixture is filtered by gravity for about 18 hours, preferably in the cold. If appreciable precipitate develops in the filtrate, it should be refiltered. A sample of the filtrate should remain practically clear when heated to 80° for 5 minutes. Additional filtrate may be obtained by pressing the precipitate, but it is usually discarded. The inhibitor is precipitated by adding 2 to 2½ volumes of 99 per cent acetone to the filtrate. The character of the precipitate seems to vary. If it settles rapidly (15 to 30 minutes), the supernatant is decanted, even though it is somewhat cloudy. Additional acetone is added

to the sediment, which is finally collected by gravity filtration. If the precipitate does not settle, it should be collected by filtration on a Büchner funnel with Celite filter aid. The filter cake is suspended in a volume of water equivalent to 0.05 to 0.1 the volume of the filtrate. The Celite is removed by filtration and the inhibitor precipitated with acetone. In this case it should settle rapidly and permit decanting. The inhibitor should be dissolved in water adjusted to pH 4.5 and dialyzed to remove trichloroacetic acid, which otherwise tends to precipitate with ovomucoid as a salt. The inhibitor is finally precipitated from the dialysate, washed with acetone and ether, and air-dried at room temperature. The yield of inhibitor is 40 to 50 per cent of the total inhibitor of egg white on an activity basis and is about 0.7 gm. dry weight per 100 ml. of egg white. The preparation contains about 8.5 inhibitor units per mg. This method is satisfactory for preparing inhibitor from egg white from which lysozyme has been recovered by the direct crystallization procedure of Alderton and Fevold (17).

*Failure to Separate Antitryptic Activity from Ovomucoid by Fractionation*—Four lines of evidence indicate that the ovomucoid fraction of egg white is identical with antitrypsin: (a) All inhibitor preparations with high activity (8.5 to 9.5 units per mg.) were obtained by methods that yield ovomucoid; (b) all ovomucoid preparations that we tested, except those prepared by severe heat treatment; possess this same high activity; (c) the activity of 8.5 to 9.5 units per mg. for the ovomucoid corresponds to 11.8 to 10.5 per cent ovomucoid in dry egg white, which approximates the percentages, 11.7 and 12.6, reported by Sørensen (15) and by Longworth *et al.* (1). Differences of this order might represent experimental error or true differences in the composition of eggs, since variations in the protein composition of egg white have been noted (1). Although a systematic study of the variation in antitryptic activity and ovomucoid has not been made, incidental observations indicate that antitryptic activity of egg white does not vary more than 20 per cent, and possibly much less; (d) a limited electrophoresis study made by W. H. Ward of this Laboratory indicated that the major, if not the sole, component of one of our purified inhibitor preparations was ovomucoid. That is, the boundary migrated at essentially the same rate as was reported by Longworth *et al.* (1) for ovomucoid.

In spite of these strong indications that the inhibitor and ovomucoid are identical, attempts were made to separate the inhibitor from ovomucoid by fractionation. These attempts were especially important in view of the report by Balls and Swenson (4) that one of their preparations of inhibitor had 150 times the activity of dried thin white compared with the maximum we have observed of about 9 times. Attempts to repeat the preparation of highly active inhibitor by the methods used by Balls and Swenson have uniformly given, in our hands, preparations of  $8\frac{1}{2}$  to 9 times the activity of dried

egg white. As previously mentioned, the results of these and earlier workers may have been influenced by their use of enzyme preparations containing in large part enzyme precursors and enterokinase, whereas this study concerns only the direct inhibition of trypsin purified by crystallization.

A 13 per cent solution of ovomucoid was fractionated with acetone and also with ammonium sulfate at pH 4.3, the isoelectric point of ovomucoid (1). Precipitation with acetone began when slightly more than 1½ volumes of acetone had been added. The acetone precipitates were washed with acetone and dried. The first ammonium sulfate fraction was separated at about 2.6 M ammonium sulfate (about 0.65 saturation). The ammonium sulfate precipitates were dissolved in water, dialyzed until sulfate-free, precipitated with acetone, and dried. The yields and activities of these fractions are given in Table II. The small differences in activities for the several fractions are not significant. These results show that antitryptic

TABLE II  
*Fractional Precipitation of Antitrypsin (Ovomucoid) with Acetone and Ammonium Sulfate*

Fraction	Acetone fractionation		(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> fractionation	
	Yield	Activity	Yield	Activity
	<i>per cent</i>	<i>units per mg.</i>	<i>per cent</i>	<i>units per mg.</i>
Original .....		9.0		9.0
1st .....	50	9.1	19	9.5
2nd .....	26	9.2	27	9.4
3rd .....	13	9.5	13	9.0
Handling loss .....	11		41*	

\* The loss in this experiment was unusually high.

activity is not readily separable from ovomucoid by fractional precipitation, which, of course, would be the case if antitryptic activity were indeed a characteristic of "native" ovomucoid. Subsequently, in this paper the terms "active ovomucoid" and "inactive ovomucoid" will be used, respectively, to designate ovomucoid with high antitryptic activity ("native" ovomucoid) and ovomucoid with little or no antitryptic activity ("denatured" ovomucoid; see below).

*Similarity of Chemical and Physical Characteristics of "Active" Ovomucoid and Ovomucoid Described in Literature*—Ovomucoid as generally described in the literature contains 11.7 to 12.7 per cent nitrogen, 2.2 to 2.5 per cent sulfur, and 20 to 25 per cent carbohydrate (mannose plus hexosamine), and has an optical rotation of  $-55^{\circ}$  to  $-71^{\circ}$  (see Meyer (18, 19) and Table III). The data of Table III, which are assembled in inverse chronological order except for those of Balls and Swenson, show that chemically and

TABLE III

Comparison of Analytical Data on "Active" Ovomucoid with Analytical Data on Ovomucoid As Reported in Literature

References	Yield (solids basis) per cent	Inhibitor activity*	Nitrogen per cent	Amino nitrogen per cent	Sulfur per cent	Carbohydrate (as glucose) per cent	Molecular weight gm.	Optical rotation $\sigma_D$ degrees
Present work								
Balls and Swenson (4)	7†	8.8	13.3 ± 0.2	0.7	2.2	21.6	28,800	-56
Stacey and Wooley (20)	4	26	10.6	0.55	2.03			-55
	10		12.5					-57
			12.7					-57
Gurin and Hood (21)						25.0§		
Meyer (18)			11.1-11.8			22-26		-65 to -70
Young (22)			12.7		0.89			
			12.6		1.36	23.7		
Karlberg (23)								
Sørensen (15)	11.7†		12.7					
Sevag (24)	8		11.7	0.8	2.47		49,300 (pH 2.8)	-60 (pH 2) -62 ( " 7) -56 ( " 10.5)
Mazza (25)								
McFarlane <i>et al.</i> (26)			13.4		2.3			
Needham (27)			12.7					
Mörner (28)	8		12.5		2.27	11.5		-71
Bywaters (29)			12.4		2.2			
Langstein (30)			12.4		2.19			
Osborne and Campbell (31)			12.7		2.38			-61

\* Relative to dry egg white.

† The theoretical yield, based on 8.8 as the activity of pure inhibitor, would be 11.4 per cent.

‡ This value was obtained by converting Sørensen's value of 12.7 per cent, representing per cent of proteins, to a total solids basis by multiplying by 0.925.

§ This total carbohydrate figure was calculated from the report that ovomucoid contains 12.5 per cent mannose and a 1:1 ratio of mannose and glucosamine.

physically ovomucoid is, in general, similar to inhibitor preparations made by Balls and Swenson and by the present authors. The low sulfur figures reported by Young and the low carbohydrate figure by Needham seem to be in error. Our values for carbohydrate content of active ovomucoid is given as  $\leq 21.6$  per cent, because an error is introduced in the cyanide method described by Militzer when large amounts of cystine are present. The observed value corresponded to 26.4 per cent carbohydrate (as glucose) which yielded a minimum value of 21.6 per cent after correction for cystine. The correction was made by assuming that all of the cystine reacted with cyanide during the alkaline titration. The true value, of course, would be larger than 21.6 per cent by the degree to which cystine failed to react with the cyanide. The nitrogen content of 10.6 per cent reported by Balls and Swenson is appreciably lower than the other values. In our hands the method of Balls and Swenson yielded ovomucoid having 8.7 units of activity per mg. and a nitrogen content of 11.9 per cent. Our most active ovomucoid preparation had a nitrogen content of 13.3 ( $\pm 0.2$ ) per cent. The molecular weight of 29,000 found for ovomucoid in this work is much lower than the 49,300 reported by Mazza (25). Because of inability to obtain his original publication, details of his method have not been available to us. Since Mazza may have worked with ovomucoid prepared by a heating process, the molecular weight of heat-inactivated ovomucoid is of interest. Determination of the molecular weight of inactivated ovomucoid, however, revealed no significant difference from that of fully active ovomucoid (27,900 compared with 28,800). Other characteristics that were not significantly altered by heat inactivation include acetyl content (range 4.5 to 5.2 per cent), optical rotation, and nitrogen content.

The ovomucoid prepared in this work contained the following percentage amounts of various amino acids; cystine 6.4, tyrosine 4.9, tryptophan  $\approx 0.3$ . Earlier values reported for ovomucoid are 4.8 to 5.4 (26) and 4.0 (22) per cent cystine, 3.3 to 3.6 per cent tyrosine (26), and 1.6 to 2.0 per cent tryptophan (26). Baernstein (32) reported 1.7 per cent methionine in ovomucoid. This methionine value and the cystine value (6.4 per cent) correspond to 91 per cent of the sulfur (2.2 per cent) found in ovomucoid.

Active and inactive ovomucoid gave a negative nitroprusside test in the absence of alkaline cyanide, which indicates the absence of free  $\text{—SH}$  groups in both active and inactive ovomucoid. A weak but definitely positive nitroprusside test was obtained for active ovomucoid in the presence of alkaline cyanide, which indicates the presence of "free"  $\text{S—S}$  groups. The test with inactive ovomucoid was about 5- to 10-fold stronger than with active ovomucoid. This, of course, indicates that "denaturation" as ordinarily understood accompanies inactivation (see the section on denaturation).

The characteristics of ovomucoid with high antitryptic activity (Balls and Swenson (4) and our preparation) do not appear to differ significantly from the characteristics reported in the literature for ovomucoid. Characteristics that would distinguish between the native and denatured state have not been reported previously for ovomucoid.

*"Denaturation" and Inactivation of Ovomucoid*—Although the antitryptic activity of ovomucoid is destroyed by heating in solution, it is much more stable to heat than are most proteins. Like papain (33), it is stable in 9 M urea. Table IV and Fig. 2 give data for heat lability and urea stability of active ovomucoid. Table IV also shows that a "3 minute egg" contains about two-thirds and a "10-minute egg" about one-fourth of the original inhibitor activity. Although the heat inactivation of ovomucoid does not appear to be a function of pH between pH 3 and 7 (Fig. 2), the activity is rapidly lost at 80° at pH 9. This is in line with the alkali lability of the inhibitor shown by Balls and Swenson (4), and explains the loss in activity that occurs on boiling shell eggs, since the pH of the white is generally above 8.5. It also agrees with the report of Delezenne and Pozerski (6) that the inhibiting action was largely destroyed by heating egg white to 70° for 30 minutes.

Ovomucoid in dilute solution is not precipitated by hot 5 per cent trichloroacetic acid; nor does it become insoluble at the isoelectric point when its activity is completely destroyed by heat. However, two criteria besides loss of activity show that ovomucoid is "denatured" by heating in solution. First, the intensity of the nitroprusside test in the presence of cyanide is 5- to 10-fold greater for the inactive than for the active ovomucoid. Second, active ovomucoid is practically resistant to hydrolysis by chymotrypsin, whereas inactive ovomucoid is rapidly hydrolyzed by chymotrypsin (Table V). This resistance is due only in part to inhibition of chymotrypsin by active ovomucoid. Thus, although chymotrypsin appears to be markedly inhibited by active ovomucoid at the concentrations cited in Table V, the inhibition is not sufficient to prevent hydrolysis of inactive ovomucoid in the presence of active ovomucoid. Similar results were obtained with papain. Chymotrypsin did not cause a decrease in inhibitor activity in either of the runs where active inhibitor was present. Since denatured proteins are generally more rapidly hydrolyzed by enzymes than are native proteins (34, 35), the digestibility of heat-treated ovomucoid constitutes evidence that ovomucoid is denatured by heat. The extent of hydrolysis of inactive ovomucoid (7 per cent of the estimated number of peptide bonds) indicated that a major component in the preparation is being hydrolyzed.

*Stoichiometric Relation between Trypsin and Ovomucoid at 50 Per Cent Inhibition Level*—Table VI shows that somewhat less than 1 molecule of active ovomucoid causes 50 per cent inhibition of 1 molecule of trypsin.

TABLE IV  
Stability of Antitrypsin to Urea and Heat

Treatment*	Residual activity
	<i>per cent</i>
9 M urea, 25°, 18 hrs.....	>90
9 " " 80°, ½ hr.....	>90
9 " " 100°, ¼ ".....	86
9 " " 100°, ½ ".....	66
pH 3, 80° (H <sub>2</sub> O), ½ hr.....	>90
" 6, 80° " ½ ".....	>90
" 7, 80° " ½ ".....	>90
" 9, 80° " ½ ".....	<10
3 min. egg.....	66
10 " ".....	25

\* The urea solutions, which were unbuffered, had a pH of about 4. The aqueous solutions were buffered with 0.1 N acetate at pH 3 and 6 and with 0.1 N borate at pH 9. The inhibitor concentration was 0.1 per cent in all cases. About 30 seconds were required to reach the indicated temperature and to lower the temperature to 20° after heating. Heating was carried out by manual rotation of a test-tube which contained the antitrypsin solution in a water bath, and cooling by rotation of the tube in an ice bath.

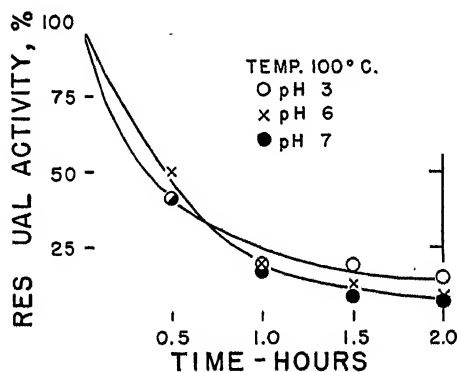
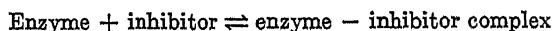


FIG. 2. Destruction of antitryptic activity at pH 3, 6, and 7 at 100°

The finding that proportionately more ovomucoid is required to cause this inhibition at lower trypsin concentrations than at the higher concentrations is consistent with the mechanism represented by the following equation:



The constancy of the dissociation constants reported in Table VI indicates that this mechanism is acceptable quantitatively. If one inhibitor mole-



cule combined with several enzyme molecules, the  $K$  values would vary with enzyme concentration. The report by Young (36) that ovomucoid exhibits only one boundary in the ultracentrifuge makes it difficult to see how the inhibition of trypsin by ovomucoid could be due to a small amount (5

TABLE V  
*Hydrolysis of Active and Inactive Ovomucoid by Chymotrypsin*

Time	$\Delta$ ml. 0.02 N NaOH*		
	100 mg. active ovomucoid	100 mg. inactive ovomucoid†	Active + inactive ovomucoid
<i>hr.</i>			
$\frac{1}{2}$	0.00	1.08	0.20
3	0.10	2.22	0.65
24	0.07	2.50	1.15

\* Hydrolysis was carried out at pH 7.8 with 1 mg. of chymotrypsin protein in a total volume of 3 ml. The  $\Delta$  values determined by a formol titration procedure correspond to increases in carboxyl groups.

† The "inactive" ovomucoid was about 95 per cent inactive.

TABLE VI  
*Stoichiometric Relation between Trypsin and Ovomucoid at 50 Per Cent Inhibition Level*

Trypsin	Ovomucoid giving 50 % inhibition*	Dissociation constant $K_{\dagger}$
<i>moles per l. <math>\times 10^3</math></i>	<i>moles per l. <math>\times 10^3</math></i>	<i>moles per l. <math>\times 10^3</math></i>
76	55	17
95	63	16
129	83	18
139	85	16

\*The values in this column were obtained by interpolation from a smooth curve relating inhibition and ovomucoid concentration.

† In these experiments the enzyme and inhibitor were allowed to stand together for 10 minutes in 1 ml. of water before being added to the 5 ml. of hemoglobin substrate. A 10 minute digestion period was used. The mm per liter were obtained by multiplying the mm per reaction mixture by 1000/6. Within experimental error, the inhibition is independent of time of digestion when the preliminary standing technique is used, but is markedly dependent (increases greatly) on time of digestion when the inhibitor is added to the substrate before the enzyme is added.

to 10 per cent) of a low molecular weight, yet non-dialyzable impurity in ovomucoid.

*Miscellaneous Observations*—Antitryptic activity of egg white is decreased by heating it in the dry state. Samples of lyophilized egg white lost 40, 94, and >99 per cent of their antitryptic activity when heated for 18 hours at

60°, 110°, and 140°, respectively. The results with these samples, which were supplied by D. K. Mecham and H. S. Olcott of this Laboratory, constitute additional evidence that marked changes occur in proteins heated in the dry state (Mecham and Olcott (37)).

Ovomucoid prepared as indicated previously shows marked gelling properties under certain conditions. A 20 per cent solution of ovomucoid in distilled water will gel in several hours at room temperature if acidified with a few drops of 3.0 M trichloroacetic acid. The resulting product is transparent and will dissolve slowly in water. A 20 per cent aqueous solution will also gel in alkali (pH 8 to 10) if placed in a boiling water bath for 5 to 15 minutes. However, a 20 per cent solution made acid with HCl to pH 2.0 to 3.0 will not gel in boiling water even after 3½ hours. The gel formed by heat in alkaline solution is insoluble in water. In general, gelling results in partial loss of inhibitor activity. For example, 37 per cent of the activity was lost when gelation was brought about with trichloroacetic acid. If conditions are sufficiently drastic, complete inactivation will result.

#### DISCUSSION

There is no reason to doubt that the trypsin inhibitor preparations made by Balls and Swenson (4) and by us are ovomucoid (*cf.* Meyer *et al.* (5)). Since ovomucoid has not been shown to be a single substance, it would be conceivable that the inhibitor is only a small fraction of what is usually recognized as ovomucoid. That this is not the case is indicated by the agreement of the antitryptic activity and ovomucoid yields, by failure of several attempts at fractionation, by the electrophoretic behavior of the preparation, and by the apparent molecule for molecule inhibition of trypsin by ovomucoid. It will be recalled that a similar stoichiometric relation was found for beef pancreas-trypsin inhibitor and trypsin (38). Electrophoretically, active ovomucoid appeared to be homogeneous, though it is possible that more extensive investigation would reveal a spreading boundary such as Longworth *et al.* (1) reported. There was no evidence of three boundaries as reported by Young for ovomucoid at 2.7 per cent concentration. A reversible spreading boundary as found by Longworth would indicate that ovomucoid is electrophoretically inhomogeneous. On the other hand, the single boundary found by ultracentrifugation (36), together with the molecule for molecule inhibition of trypsin by ovomucoid, indicates that at least the majority, if not all of the molecular species (if there is more than one), possess antitryptic activity.

Kunitz (39) noted that soy bean trypsin inhibitor differs markedly in physical and chemical properties from the beef pancreas trypsin inhibitor. It now appears that the egg white trypsin inhibitor (*i.e.* active ovomucoid) differs markedly from both the pancreas and soy bean trypsin inhibitors.

Thus, the inhibitor from pancreas has a molecular weight of about 6000, passes slowly through cellophane membranes, is not precipitated by hot or cold 2.5 per cent trichloroacetic acid, and contains about 11 per cent nitrogen (38); the inhibitor from soy bean does not diffuse through cellophane membranes, is precipitated by trichloroacetic acid, and contains 16 per cent nitrogen and no carbohydrate (39), while the inhibitor from egg white has a molecular weight of about 29,000, does not diffuse through cellophane membranes, is not precipitated by hot or cold trichloroacetic acid (a noteworthy fact, in view of the molecular weight), contains 13 per cent nitrogen, and about 25 per cent carbohydrate. Both the pancreatic and egg white trypsin inhibitors are relatively stable to heat compared with the soy bean inhibitor.

Ovomucoid is the fourth protein of egg white to be shown to have biological activity. Avidin, as is well known, combines with biotin in such a way that it causes biotin deficiency if present in the diet in sufficient quantity (40); lysozyme has been shown recently (41) to be identical with the globulin G<sub>1</sub> described by Longsworth *et al.* (1); and conalbumin has been shown recently to be the component of egg white that combines so strongly with iron that it causes iron deficiency for the growth of certain microorganisms (42, 43). All four of these proteins have in common the property of limiting biological activity, and it therefore seems likely, as has been suggested for lysozyme many times, that they all play a part in natural resistance of the egg to microbial infestation. These proteins may also play a part in the prevention of rapid autolysis of eggs.

While discussing biological activity it should be mentioned that recent reports indicate that soy bean antitrypsin causes growth retardation of animals (44-46) and acts as a blood anticoagulant (47); also, high concentrations of pancreatic antitrypsin, but not of soy bean antitrypsin, exhibit *in vitro* antibiotic activity toward  $\beta$ -hemolytic streptococci, *Staphylococcus aureus*, and *Escherichia coli* (48). Both of these antitrypsins inhibit fibrinolysis by  $\beta$ -hemolytic streptococci fibrinolysin. Grob (49) earlier concluded that antitrypsin in low concentration retards bacterial growth through prevention of proteolysis in the medium. Native ovomucoid appears suitable for investigation of the antibiotic, anticoagulant, or growth-retarding effect of egg white trypsin inhibitor.

Since egg yolk contains very little trypsin inhibitor activity, it would appear possible to develop a simple method for determining the amount of egg white present as a contaminant in commercial yolk preparations by determining their antitryptic activity. Cook and Mehlenbacher (50) recently developed a method for determining the degree of contamination of egg white by yolk with cholesterol as an index.

## SUMMARY

Antitryptic activity is equally distributed in the thin and thick white of the egg. Egg yolk contains about 0.4 per cent as much antitryptic activity as the white (dry weight basis).

The antitryptic activity of egg white appeared quantitatively in the ovomucoid fraction of egg white when it was prepared without heat treatment. Attempts to fractionate ovomucoid into components with high and low antitryptic activity failed. Limited electrophoresis runs failed to reveal the presence of protein other than ovomucoid. Less than 1 molecule of ovomucoid is required to cause 50 per cent inhibition of 1 molecule of trypsin. Heat-denatured ovomucoid has little or no antitryptic activity. Quantitative interpretation of these findings leave little doubt that antitryptic activity is a characteristic of native ovomucoid.

Native ovomucoid is completely resistant to hydrolysis by chymotrypsin, which it only partially inhibits, whereas heat-denatured ovomucoid, which possesses little or no antitryptic activity, is readily hydrolyzed by chymotrypsin even in the presence of active ovomucoid. The test for S—S groups (nitroprusside with cyanide) is about five times as strong for denatured as for native ovomucoid. Both native and denatured ovomucoid are very soluble in water.

Ovomucoid, prepared as described in this paper, has an average molecular weight of 29,000 and an optical rotation of  $-56^\circ$  ( $[\alpha]_D^{20}$ ). Analysis revealed the following percentage composition: total nitrogen 13.3, amino nitrogen 0.7, acetyl, 4.5 to 5.2, sulfur 2.2, cystine 6.4, tyrosine 4.9, tryptophan,  $\approx 0.3$ , carbohydrate,  $\approx 21.6$  (as glucose), SH, none detectable.

A method is given for preparing ovomucoid without resort to heat treatment.

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# FORMATION AND LOSS OF CYSTEINE DURING ACID HYDROLYSIS OF PROTEINS. RÔLE OF TRYPTOPHAN

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## Cysteine

Brand and Kassell (1) showed that an acid hydrolysate of chymotrypsinogen contained a considerable amount of cysteine (1.3 per cent), although sulfhydryl groups could not be demonstrated in the intact protein, even after denaturation (1, 2). The meaning of this observation was not clear. Recently it was observed in this Laboratory that an acid hydrolysate of lysozyme, which also lacks detectable sulfhydryl groups, contained cysteine. The relatively high tryptophan contents of these proteins (6.0 and 8.0 per cent, respectively) suggested to us that this amino acid might be involved in the formation of cysteine. The experiments to be described confirmed the hypothesis that tryptophan causes a reduction of cystine under the conditions used for the acid hydrolysis of proteins.

In addition some further factors responsible for the amounts of cysteine determinable in protein hydrolysates have been elucidated. Foreexample, it has been shown that there is slight but detectable *formation* of cysteine when cystine alone is heated with acids. Such a reaction may account for the 0.6 per cent cysteine content of an insulin hydrolysate recorded by Brand (3). On the other hand, Halwer and Nutting (4) have recently called attention to the *disappearance* of cysteine from acid hydrolysates of proteins. Cysteine, added either before or after acid hydrolysis, could not be quantitatively recovered, even though oxidative destruction (by air) was minimized. It has now been shown that the constituent responsible may be pyruvic acid, a substance that is present in protein hydrolysates from the decomposition of serine and possibly cystine peptides.

The autoxidizability of cysteine in hydrochloric acid solution in the presence of copper ions has also been studied.

## EXPERIMENTAL

*Materials and Methods*—The amino acids used were commercial products. Crystalline insulin was kindly furnished by Eli Lilly and Company, gramicidin by the Wallerstein Company, lysozyme by G. Alderton and H. L. Fevold, and chymotrypsinogen by E. F. Jansen. Phosvitin is a phospho-

\* Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture.



protein containing 10.3 per cent phosphorus, present as orthophosphate esters of hydroxy amino acids.<sup>1</sup> The phosphorylated sericin (8.4 per cent phosphorus) was prepared by treating sericin with phosphoric acid containing excess phosphorus pentoxide.<sup>2</sup>

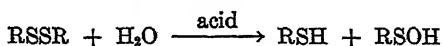
For some experiments, concentrated hydrochloric acid was diluted with an equal volume of water and distilled as recommended by Hess and Sullivan (5) to remove oxidizing material. No benefit was noted. The pyruvic acid was a commercial sample, redistilled before use.

In experiments designed to demonstrate the formation of cysteine, or to minimize the oxidative destruction of tryptophan, the reactions were carried out in Thunberg tubes (Silicone lubricant) that had been evacuated (water pump), filled with carbon dioxide, and then reevacuated.

Cysteine in the presence of excess cystine was determined by titration with 0.002 or 0.005 M *p*-chloromercuribenzoate with nitroprusside as an outside indicator (6, 7). The more specific Sullivan reaction (8) (but without prior reduction with either cyanide or sodium amalgam) was used in some experiments to demonstrate that cysteine rather than other reducing agents was being measured. For the experiments in which the disappearance of cysteine was investigated, the amino acid was determined by Folin's uric acid reagent (9) according to the procedure recommended by Anson (10).

✓ Tryptophan was determined by the excellent colorimetric method of Horn and Jones (11).

*Formation of Cysteine*—As shown by the data in Table I, some cysteine is obtained merely by heating cystine in strong acid, probably from hydrolysis of the disulfide bond. More is formed in sulfuric acid than in hydro-



chloric acid and more at 125° than at 100°. The *absolute* amounts obtained, however, are insignificant, except for those observed with sulfuric acid at 125°.

In the presence of added tryptophan, an appreciable fraction (20 to 30 per cent) of the cystine is reduced to cysteine at 100–125° in 7 N hydrochloric acid, but the reaction is minimal in sulfuric acid at 100°. The data are presented in Table II. In general the percentage of the cystine reduced increased with increasing amounts of tryptophan but stoichiometric relationships were not found.

The same reactions were observed when cystine was present in the form of insulin, which contains approximately 12 per cent cystine and no tryptophan, or when tryptophan was supplied in the form of gramicidin, which

<sup>1</sup> Mecham, D. K., and Olcott, H. S., manuscript in preparation.

<sup>2</sup> Ferrel, R. E., Olcott, H. S., and Fraenkel-Conrat, H., manuscript in preparation.

contains 40 per cent tryptophan but no cystine (Table III). When insulin was hydrolyzed in the presence of 10 per cent of its weight of tryptophan,

TABLE I  
*Formation of Cystine from Cystine in Acid Solution\**

Acid	Temperature†	Cystine	Cystine found‡
	°C.	mg.	per cent
Hydrochloric (6-7 N).....	100	10	0.3
“ (6-7 “).....	100	50	0.5
“ (6-7 “).....	125	10	3.5
Sulfuric (6 N).....	100	10	2.1
“ (6 “).....	100	50	2.2
“ (6 “).....	125	10	15.0

\* 18 hours in evacuated Thunberg tubes, total volume 1 ml.

† Air oven.

‡ As percentage of cystine present.

TABLE II  
*Formation of Cystine from Cystine by Reaction with Tryptophan under Conditions Used for Acid Hydrolysis of Proteins\**

Cystine†	Tryptophan‡	Conditions of treatment	Cystine found§	Tryptophan destroyed
mg. per ml.	mg. per ml.		per cent	per cent
2.5	2.5	7 N HCl, 100°, 18 hrs.	25	43
10	10	7 “ “ 100°, 18 “	26	52
2.5	2.5	7 “ “ 110°, 18 “	37	56
2.5	50	7 “ “ 110°, 18 “	50	53
5.0	5.0	7 “ “ 110°, 18 “	20	42
10.0	2.5	7 “ “ 110°, 18 “	16	92
10.0	10.0	7 “ “ 110°, 18 “	32	47
10.0	20.0	7 “ “ 110°, 18 “	40	55
10.0	1.0	7 “ “ 125°, 6 “	4	
10.0	3.0	7 “ “ 125°, 6 “	8	
10.0	10.0	7 “ “ 125°, 6 “	15	
10.0	10.0	6 “ H <sub>2</sub> SO <sub>4</sub> , 100°, 18 “	2.5	6
10.0	10.0	6 “ “ 125°, 18 “	26	42

\* In evacuated Thunberg tubes.

† For cystine controls, see Table I.

‡ For tryptophan controls, see Table VIII.

§ As per cent of cystine used.

the hydrolysate contained cysteine equivalent to 2.1 per cent of the insulin used.

The data obtained with lysozyme and chymotrypsinogen, proteins containing tryptophan and cystine, but no detectable sulfhydryl groups, are shown in Table IV. They indicate that the same reactions occur when both

TABLE III  
*Formation of Cysteine during Hydrolysis of Amino Acid and Protein Mixtures\**

Mixture	Cystine equivalent present	Trypto- phan equivalent present	Cysteine found	
	mg.	mg.	mg.	per cent†
50 mg. insulin.....	6	0	0	0
40 " " + 5 mg. tryptophan.....	5	5	0.5 Ca.	10 Ca.
50 " " + 25 " gramicidin.....	6	10	1.1	19
100 " " + 10 " tryptophan.....	12	10	2.1	18
5 mg. cystine + 12.5 mg. gramicidin....	5	5	0.5 Ca.	10 Ca.
10 " " + 25 mg. gramicidin.....	10	10	2.4	24

\* Hydrolyzed for 18 hours at 100° with 2 ml. of 7 N hydrochloric acid in evacuated Thunberg tubes.

† As percentage of cystine present.

TABLE IV  
*Effect of Acid Hydrolysis on Apparent Cysteine and Tryptophan Contents of Lysozyme and Chymotrypsinogen\**

Protein	Method of hydrolysis†	Apparent cysteine content	Apparent tryptophan content
		per cent	per cent
Lysozyme	6 N HCl, 100°, 18 hrs.	1.2	2.7
	6 " H <sub>2</sub> SO <sub>4</sub> , 100°, 18 hrs.	0.3	5.9
	None	None‡	8.0
Chymotrypsinogen	6 N HCl, 100°, 18 hrs.	1.5	2.9
	6 " H <sub>2</sub> SO <sub>4</sub> , 100°, 18 hrs.	0.4	3.9
	None	None‡	6.0

\* Lysozyme contains approximately 5 per cent cystine, chymotrypsinogen, 4.6 per cent.

† In evacuated Thunberg tubes, 50 mg. of protein, 0.5 ml. of acid.

‡ A solution of the protein in saturated guanidine hydrochloride solution gave a negative nitroprusside test. Under the same conditions, a strong positive test was obtained with bovine serum albumin, which is known to contain 0.3 to 0.4 per cent cystine (12).

amino acids are originally present in peptide linkage, and that the presence of other amino acids does not interfere with the cystine-tryptophan reaction.

In order to determine whether other amino acids or proteins (lacking

tryptophan) could reduce cystine under the conditions of acid hydrolysis, 5 or 10 mg. samples of cystine were heated for 18 hours in 7 N hydrochloric acid at 100° or 110° with the following additions: 40 mg. of serine, 50 mg. of arginine hydrochloride, 40 mg. of threonine, 40 mg. of hydroxyproline, 20 mg. of proline, 20 mg. of tyrosine, 20 mg. of phenylalanine, 100 mg. of zein, 100 mg. of protamine sulfate, and 100 mg. of polyglutamic acid. The amounts of cysteine produced ranged from 0 to 0.18 mg., accounting for up to 1.8 per cent of the cystine. This amount of reduction is slight compared to that observed with tryptophan. When 10 mg. of cystine were heated with 100 mg. of gelatin under the same conditions as those described above, 0.2 to 0.3 mg. of cysteine was consistently produced. The constituent in gelatin responsible for this amount of reduction is not known.

*Destruction of Cysteine*—Halver and Nutting (4) considered the possibility that aldehydes might be responsible for the disappearance of cysteine from acid hydrolysates of proteins but could not obtain a positive fuchsin test in such solutions. It occurred to us that the unknown constituent might be pyruvic acid, a compound that is known to arise from the decomposition of serine (13, 14) and possibly of cystine (15) during acid hydrolysis. In the experiments outlined in Table V, pyruvic acid was found to cause the disappearance of cysteine even at room temperature in 7 N hydrochloric acid. Furthermore, hydrolysates of those proteins that contain considerable amounts of phosphate esters of serine, and which, therefore, should yield large amounts of pyruvic acid (14, 16), also were capable of reacting with added cysteine (Table V).

In further experiments it was found that many amino acids and protein hydrolysates in amounts of 50 to 100 mg. caused the loss of 5 to 25 per cent of 1 mg. of cysteine hydrochloride under the conditions used; namely, 3 days of standing in 7 N hydrochloric acid at room temperature in stoppered test-tubes. However, these losses might be due to trace contamination with heavy metals, particularly copper and iron. The former was found to be a powerful catalyst for the oxidation of cysteine in strong hydrochloric acid solution (Table V).

The disappearance of cysteine in the presence of pyruvic acid cannot be explained on the basis of oxidation catalyzed by traces of heavy metals. The pyruvic acid had been redistilled, and was as reactive in the absence as in the presence of air (Table VI).

#### DISCUSSION

In addition to those discussed by Lugg (17), the following factors have now been shown to be involved in the amounts of cysteine determinable in protein acid hydrolysates: (1) the amount of cysteine *formed* by the reaction of tryptophan with cystine and, to a lesser degree, by the acid hydrolysis of

cystine itself, and (2) the amount of cysteine *lost* by reaction with pyruvic acid.

TABLE V

*Loss of Cystine in Hydrochloric Acid Solutions\* containing Pyruvic Acid, Protein Hydrolysates,† or Copper Sulfate*

Addition	Amount	Cystine recovered
	mg.	per cent
None.....		96-100
Pyruvic acid.....	1	72
“ “ .....	4	28
“ “ .....	10	7
Formaldehyde.....	1	75
Phosvitin hydrolysate.....	20	30
“ “ .....	100	0
Phosphorylated sericin hydrolysate.....	20	12
Sericin hydrolysate.....	100	68
Copper sulfate·5H <sub>2</sub> O.....	0.01	89
“ “ .....	0.1	8‡
“ “ .....	0.1 ( <i>in vacuo</i> )	71
“ “ .....	1.0	0
Amino acids or protein hydrolysates§.....	50-100	67-95

\* The solutions contained 1 mg. of cysteine hydrochloride in 2 ml. of 7 N hydrochloric acid in stoppered test-tubes. They were held at room temperature for 3 days, then analyzed for residual cysteine. No precautions were taken to exclude air, except in the experiment with copper sulfate. Attempts to measure cysteine recoveries from evacuated Thunberg tubes in general gave less satisfactory check results, possibly due to unknown catalytic effects of the stop-cock lubricant.

† The amount of protein indicated was refluxed for 18 hours in 1 ml. of 7 N hydrochloric acid at 125°. After being cooled, 1 ml. of a solution containing 1 mg. of cysteine hydrochloride per ml. of 7 N hydrochloride was added.

‡ When 0.1 mg. of copper sulfate was added to the cysteine in the presence of 6 N sulfuric acid rather than 7 N hydrochloric acid, 80 per cent of the cysteine was present after 3 days. The surprisingly different catalytic effect of copper in the two acids was not investigated further.

§ The amino acids used were 50 mg. each of serine, tyrosine, hydroxyproline, threonine, glutamic acid, aspartic acid, arginine hydrochloride, methionine, phenylalanine, tryptophan, and tyrosine, and 100 mg. of a mixture containing 20 mg. each of glycine, alanine, valine, leucine, and proline. The hydrolysates used were prepared from 100 mg. of gelatin, protamine sulfate, polyglutamic acid, and nylon. When serine was “hydrolyzed” by the procedure used with the proteins, then added to cysteine, there was only a slightly greater loss on standing than when serine alone was used (90 per cent compared to 95 per cent). Hence, little if any pyruvic acid is present in such a preparation.

The cysteine analyses recorded for chymotrypsinogen (1.3 per cent) ((1); also Table IV) probably reflect its tryptophan content, and that obtained

with insulin (0.6 per cent) (3) is possibly due to acid hydrolysis of cystine. Brand (3) has also reported cysteine contents of other protein hydrolysates in excess of the content of SH groups determinable in the intact proteins. These need not be listed. In view of the previous discussion it appears that they do not measure accurately the cysteine contents of the proteins, and that the amount by which they deviate is difficult to assess quantitatively.

Hess and Sullivan (5) compared the <sup>\*\*\*</sup>sulphydryl contents of a series of intact proteins with the cysteine present in acid hydrolysates. There was good agreement between the two sets of data and it was concluded that the cysteine of the protein appeared quantitatively in the hydrolysate. From

TABLE VI

*Rate of Reaction of Pyruvic Acid with Cysteine in 6 N Hydrochloric Acid at Room Temperature (25°)\**

Time	Cysteine recovered
	<i>per cent</i>
0 .....	100
15 min.....	92
1 hr.....	91
4 hrs.....	83
1 day.....	65
3 days.....	39†
4 " .....	35

\* In rubber-stoppered test-tubes. The solutions contained 1 mg. of cysteine hydrochloride and 4 mg. of distilled pyruvic acid in 2 ml. of 6 N distilled hydrochloric acid.

† 41 per cent was recovered when the solution was held in an evacuated Thunberg tube.

the observations recorded above, such agreement should probably be interpreted as fortuitous, the result of compensating errors.

Several investigators have accounted quantitatively for the non-sulfate sulfur of proteins in terms of their total methionine, cystine, and cysteine sulfur contents (3, 5, 18, 19). Some proteins appear to be more vulnerable than others to the side reactions that interfere with this summation (5, 18, 20-22). Smith and Greene (20) accounted satisfactorily for the sulfur of several immune proteins but were unable to recover that of some plant globulins. Cuthbertson and Phillips (15) concluded that reaction of cysteine with pyruvic acid did not complicate their detailed analyses of the sulfur constituents of various wools. However, the evidence for this conclusion is not convincing. The possibility that condensation occurred during acid hydrolysis was not investigated. A definite fraction of the

total sulfur could not be identified as cystine, sulfhydryl, sulfate, or bromine-oxidizable sulfur. The method of hydrolysis undoubtedly plays a rôle. Foreexample, if air and catalytic amounts of heavy metals are present, the cysteine formed by reaction with tryptophan may be reoxidized to cystine before it is removed by reaction with pyruvate. At least at room temperature the latter reaction is a slow one (Table VI).

The data suggest that the reactions described would be minimized if proteins containing tryptophan were hydrolyzed at 100° with 6 N sulfuric acid *in vacuo*, and if those containing no tryptophan were hydrolyzed with 6 N hydrochloric acid under the same conditions. As an example, a sample of reduced insulin was found to contain sulfhydryl groups corresponding to 4.5 per cent cysteine both in the intact protein and in a hydrochloric acid hydrolysate (by chloromercuribenzoate titration). In general, however, determinations of the sulfhydryl groups by titrations performed on the unhydrolyzed denatured protein would appear least subject to error or artifact.<sup>3</sup>

The nature of the reaction of cystine with tryptophan in acid solution is known only from the results; namely, the formation of cysteine and disappearance of tryptophan, from which it may be inferred that the tryptophan is oxidized by the disulfide linkage of cystine. A disulfide containing no amino or carboxyl groups, namely bis(2-hydroxyethanol)disulfide, was also found to become reduced and to destroy tryptophan in acid solution (Table VII).

Schubert (24) investigated the reaction of cysteine with *pyruvic acid* in non-aqueous media. The isolated material corresponded in analyses to an addition product. The nature of the reaction in 7 N hydrochloric acid, observed in the present work, has not been investigated. However, the reaction of *formaldehyde* with cysteine in 6 N hydrochloric acid has been shown recently to lead to the formation of djenkolic acid or thiazolidinecarboxylic acid, depending upon the relative amounts of the reagents used (25). Hess and Sullivan (26) showed that the reaction product of cysteine and formaldehyde was not determinable as cysteine by any of the color reactions usually used. Other aldehydes or reactive ketones present as decomposition products of amino acids or other protein constituents might be expected to react similarly (24, 27, 28).

The acceleration of the aerobic oxidation of sulfhydryl compounds with copper has been studied in detail (29). In general, the reaction is much more rapid in alkaline than in neutral or acid solution (30, 31). However, Lugg (17) showed that ferrous iron catalyzed the oxidation of cysteine in 0.25 N hydrochloric acid. In the limited series of experiments shown in

\* The use of enzyme hydrolysates is suggested by the work of Anson (10).

Table V, oxidation occurred at room temperature in the presence of copper in hydrochloric acid. In sulfuric acid, the cysteine was considerably more stable.

TABLE VII

*Effect of Various Additives on Recovery of Tryptophan from Heated Acid Solutions\**

Addition	Weight	Acid, 6 N	Tryptophan recovered
	<i>mg.</i>		<i>per cent</i>
Cystine.....	2.5†	HCl	50
Bis(2-hydroxyethyl) disulfide.....	2.5	"	16
Serine.....	10	"	88
".....	25	"	52
Pyruvic acid.....	0.7	"	74
" ".....	1.3	"	44
" ".....	1.3	H <sub>2</sub> SO <sub>4</sub>	44
" ".....	2.5	"	16
" ".....	2.5	HCl	11
Sericin.....	10	"	72
".....	125	"	0
Phosphosericin.....	10	"	65
Phosvitin.....	5	"	68
Protamine sulfate†.....	67	"	49
" " †.....	125	"	42
Gelatin†.....	125	"	51
Zein†.....	125	"	65
Nylon†.....	125	"	80

\* 2.5 mg. of tryptophan per ml. Heated 18 hours at 100° in evacuated Thunberg tubes. Recoveries ranged from 88 to 96 per cent in tubes containing the following additions: 10 and 25 mg. of threonine, 10 and 25 mg. of hydroxyproline, and, separately, 25 mg. each of tyrosine, arginine hydrochloride, glutamic acid, and glycine. There was no destruction of tryptophan in the presence of 0.1 mg. of copper sulfate. For the controls, see Table VIII.

† See Table II for further data with cystine.

‡ Contains no tryptophan. According to Rees (23), the serine contents of protamine sulfate and gelatin are 5.0 and 3.2 per cent, respectively.

### *Tryptophan*

Lugg (32) reviewed the work of previous investigators and made further observations concerning the stability of tryptophan in acid solution. In contrast to Folin's statement (33) that tryptophan was less stable in acid than had been commonly supposed, Lugg concluded that it was more stable.

The data recorded in Tables II to IV are in accord and permit the following conclusions: In the absence of air, tryptophan is relatively stable in hot acids, either alone or in the presence of a number of amino acids (Tables



VII and VIII). However, when cystine or other disulfides are present, tryptophan is destroyed. It is also destroyed when heated in solution with pyruvic acid or proteins from which pyruvic acid may be derived. The destruction of tryptophan in acid solutions containing *carbohydrates* has been noted by many investigators since Gortner and Blish (34) first described the phenomenon.

In the light of these observations it is understandable why gramicidin, which contains no cystine or serine, can be hydrolyzed by acids without destruction of its tryptophan content (35, 36), whereas such a procedure is unsuited for most proteins.

The reaction of tryptophan with pyruvic acid, like that with carbohydrates, results in the formation of brown to black solutions and precipitates (humins), depending upon the amount of tryptophan destroyed. However,

TABLE VIII  
*Recovery of Tryptophan from Heated Acid Solutions\**

Acid	Temperature	Tryptophan recovered
	°C.	<i>per cent</i>
Hydrochloric (6-7 N)	100	95†
	125	95
Sulfuric (6-7 N)	100	95
	125	80

\* 2.5 mg. of tryptophan per ml. Heated in evacuated Thunberg tubes for 18 hours. There was no loss of tryptophan from similar solutions held at room temperature either *in vacuo* or in the presence of air.

† When air was present, less than 18 per cent of the tryptophan was determinable.

the reaction with cystine, particularly at 100°, accomplishes destruction of tryptophan without humin formation; *e.g.*, the solutions remain water-clear.

It is of interest that *tryptophan-cysteine* interactions have recently been reported as occurring during alkaline hydrolysis (37, 38). Together with the experiments reported here, they emphasize the difficulties in drawing conclusions concerning the amino acid composition of a protein from the results of analyses on hydrolysates.

#### SUMMARY

Under the conditions usually used for the acid hydrolysis of proteins (heating at 100° or 125° for 6 to 24 hours), cystine reacts with tryptophan to give cysteine. The reaction occurs much more readily in 6 to 7 N hydrochloric acid than in 6 N sulfuric acid.

Some cysteine results from the acid hydrolysis of cystine in the absence of tryptophan. The reaction, however, is only appreciable at 125° in

6 N sulfuric acid. The cysteine contents of acid hydrolysates of proteins containing no sulfhydryl groups reflect both of these reactions.

Cysteine reacts with pyruvic acid in acid solution. This reaction may explain the loss of cysteine from protein acid hydrolysates noted by Halwer and Nutting. The oxidation of cysteine in hydrochloric acid solution is catalyzed by copper salts.

Tryptophan is fairly stable in hot acid solutions in the absence of air. Both pyruvic acid and cystine cause destruction; the latter reaction occurs without the formation of humin.

The bearing of these observations on the interpretation of the amino acid composition of proteins is noted.

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INACTIVATION OF PARAMECIN ("KILLER" SUBSTANCE  
OF *PARAMECIUM AURELIA* 51, VARIETY 4) AT  
DIFFERENT HYDROGEN ION CONCENTRATIONS AND TEMPERATURES\*

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Sonneborn (1) has described a character (the "killer" character) in varieties 2 and 4 of *Paramecium aurelia*. These stocks secrete into the medium an antibiotic substance. The killer that has been most fully studied is stock 51 of variety 4 of *Paramecium aurelia*. When sensitive stocks are exposed to the culture fluid containing the antibiotic substance secreted by the killer stock 51, they develop certain characteristic changes (2). A slight hump appears after several hours on the aboral surface near the hind end of the body. This hump gradually enlarges while the anterior end of the body wastes away and the posterior part is pushed into the humped region. The animals then become smaller and spherical and finally die. In any 5 hour interfission period, the amount of paramecin liberated into the medium by the killer animals is such that the usual relation between the numbers of the killer animals that had lived in the culture fluid for that period and the number of sensitive animals killed by the fluid is 1:1 (3). This indicates that in this period 1 unit of paramecin is released by one killer animal and that 1 unit is sufficient to kill a sensitive animal. More paramecin can be released when the animals are disintegrated by repeatedly forcing a suspension of *Paramecium aurelia* through a narrow gage injection needle (4).

The manifestation of the killer character is dependent upon both a cytoplasmic and a chromosomal factor (5). Mating tests proved that the latter is a single dominant gene, designated *K*. Sensitive clones (*KK*) exist which, without the cytoplasmic factor, do not manifest the killer character, but acquire this when the factor *k* is introduced. Thereafter the clones reproduce true to form. When the cytoplasmic factor is introduced into clones homozygous for the recessive allele (*kk*), the killer character does not manifest itself; nor is the cytoplasmic factor perpetuated. In about half of the known varieties of *Paramecium aurelia* a cytoplasmic factor intervenes between the genes at each locus and their phenotypic manifesta-

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tions. Examples of hereditary traits depending upon the presence of a cytoplasmic factor are those of mating type and antigen formation (6). There exist important resemblances between the properties of the genes and the cytoplasmic factors; both are self-reproducible under appropriate conditions and both control characters. The cytoplasmic factors for different characters are discrete and separable (7). However, there is no necessary correlation between the rates of reproduction of the genes and the cytoplasmic factors (8).

Cytoplasmic factors may be of general significance. L'Héritier and co-workers (9, 10) analyzed the carbon dioxide resistance in *Drosophila* which presents in many respects a striking parallel to the results obtained on *Paramecium aurelia*. Darlington (11) and Haddow (12) emphasized the rôle played by plasma genes in animals, plastogenes in plants and viruses. These cytoplasmic systems, while probably dependent upon a chemical or even a physiological equilibrium, have an important function in cell differentiation. They constitute that part of the genetic system that is not associated with any visible bodies in the cell, although it is related to the genes. Several other instances of cytoplasmic inheritance can be cited. Rhoades (13) described a gene-induced transmissible plastid difference in maize. Experimental work by Woods and DuBuy (14, 15) suggests the possibility, as pointed out by these authors, that some phytopathogenic viruses might be related in origin to certain constituents of the cytoplasm, the mitochondria. Heston, Deringer, and Andervont (16) and Heston (17) pointed out that the gene-cytoplasmic relationship in *Paramecium aurelia* resembles in many respects the gene-milk agent relationship in mammary tumor development in mice.

Paramecin, besides being the end-product of a gene-cytoplasmic factor relationship, is the first antibiotic known to be produced in an animal cell. It will be of great value for the understanding of its mode of production and its mode of action to have this compound identified as to its chemical constitution. It will then be possible to investigate the relationship between paramecin and the cytoplasmic factor and ultimately the whole hereditary system of gene-cytoplasmic factor-paramecin.

We wish to report here some preliminary investigations on the nature of paramecin.

#### EXPERIMENTAL

*Method of Testing. Preparation of Test Solutions*—*Paramecium aurelia* 51, variety 4, were grown in Erlenmeyer flasks on a lettuce infusion, inoculated with the bacterium *Aerobacter aerogenes* (18), at the rate of two fissions per day. When about 2000 animals per cc. were present, the concentration was increased approximately 10-fold by filtering the culture through a Berkefeld filter after clarification by filtering through a Gooch crucible

lined with a loose layer of cotton (19). At this point the number of animals per cc. was determined by counting the *Paramecia* present in 1 cc. of an appropriate dilution. Final concentration was achieved by centrifuging the suspension in an angle centrifuge at 2000 R.P.M. for 10 minutes. The animals present in 1 cc. of the supernatant liquid were counted and this

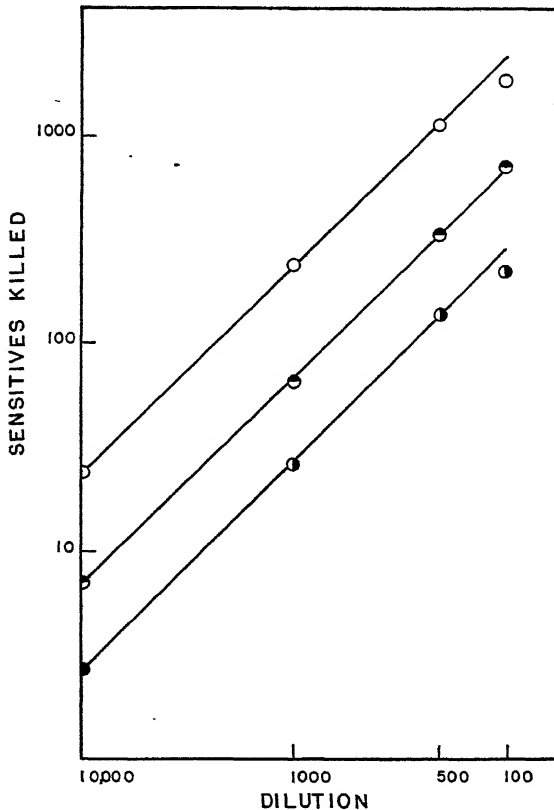


FIG. 1. Numbers of sensitive *Paramecium aurelia* killed by three different preparations of a killer paste at increasing dilutions of each paste. O, initial activity of the paste 20,000 units of paramycin per cc., ◐, initial activity of the paste 70,000 units of paramycin per cc., and ●, initial activity of the paste 270,000 units of paramycin per cc.

figure subtracted from the original count. This figure, multiplied by the number of cc. of suspension that had been centrifuged, was taken to represent the number of animals used for the determination. The packed animals were then suspended in 10 cc. of phosphate buffer<sup>1</sup> of pH 7 and

<sup>1</sup> The buffer was prepared by mixing 6 volumes of 0.25 M disodium phosphate solution with 0.25 M monosodium phosphate solution, until a pH of 7 was obtained when diluted to 0.01 M.

subsequently disintegrated by forcing the suspension through a narrow gage (No. 27) injection needle. Ten to fifteen passages through the needle were usually sufficient for a complete disintegration of all the animals present. The resulting paste was always subjected to a careful examination under a low power microscope and any non-disintegrated animals which might be present were removed. Two 4 cc. portions of this paste were pipetted into test-tubes, standing in a constant temperature water bath. The pH in one of the tubes was adjusted to the desired value with hydrochloric acid

TABLE I  
*Inactivation of Crude Paramecin Preparations at Different  
Hydrogen Ion Concentrations at 30°*

pH	No. of determinations	Per cent activity							K per min. <sup>-1</sup> (1st order reaction)
		0 min.	10 min.	20 min.	30 min.	40 min.	50 min.	60 min.	
1.0	1	0	0	0	0	0	0	0	
2.0	1	0	0	0	0	0	0	0	
3.0	1	0	0	0	0	0	0	0	
4.0	1	0	0	0	0	0	0	0	
5.0	1	0	0	0	0	0	0	0	
6.0	2	100.0	70.7	51.5	35.5	28.8	16.3	13.0	0.0377
7.0	10	100.0 ±0.0*	90.2 ±0.9*	80.9 ±1.3*	70.0 ±1.4*	61.7 ±1.7*	55.9 ±1.5*	49.7 ±1.7*	0.0119
7.5	1	100.0	94.0	89.3	83.0	78.0	75.0	67.8	0.0062
8.0	4	100.0	96.0 (97.3)	93.2 (94.5)	89.5 (92.0)	86.5 (87.8)	84.5 (85.5)	82.3 (83.0)	0.0033
			-95.0)†	-93.0)†	-88.0)†	-84.5)†	-82.0)†	-80.5)†	
8.5	1	100.0	97.1	94.2	92.7	92.0	86.1	84.7	0.0028
9.0	1	100.0	89.0	76.0	62.6	53.0	45.0	39.0	0.0154
9.5	1	0	0	0	0	0	0	0	
10.0	1	0	0	0	0	0	0	0	
11.0	1	0	0	0	0	0	0	0	

\* Per cent activity ± standard error.

† The figures in parentheses represent the range.

or sodium hydroxide solutions, the pH being measured with the glass electrode. A rough adjustment was made first with 0.1 N solutions. The acid or alkali was added very slowly under constant stirring, in order to prevent local increases of the pH above the desired value. Final adjustment was made with 0.01 N acid or alkali. An equivalent amount of buffer solution was added to the other tube. The temperature of the bath was maintained at 30° with a fluctuation of ±0.02°.

*The Test*—Immediately after the volume of the paste in the second test-tube was adjusted, the first sample was removed from both tubes. This

time was arbitrarily taken as zero time. The sample (0.05 cc.) was immediately diluted to 25 cc. with the original buffer solution. Each 10 minutes a sample was removed, the last one being taken 60 minutes after zero time. Occasional checks did not show a change of pH in any of the determinations at the end of 1 hour. The pH of the dilutions was not significantly different from the original pH of the buffer solution. The activity of the dilutions was tested as follows. A suspension of sensitive animals (*Paramecium aurelia* 31, variety 8) was grown in the same manner

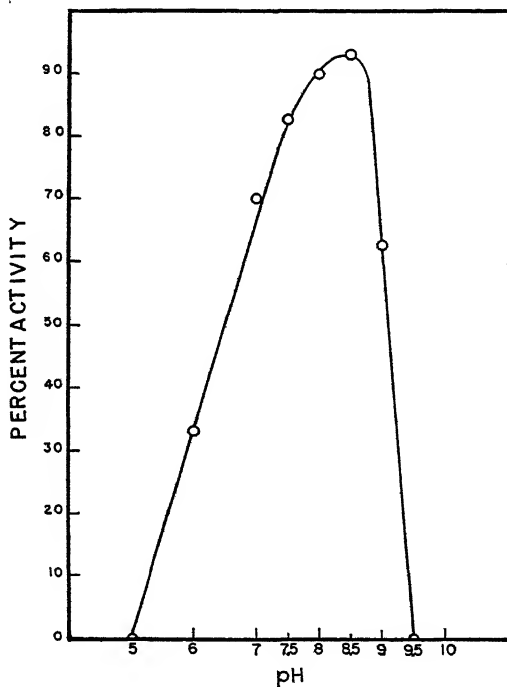


FIG. 2. The influence of the hydrogen ion concentration upon the stability of paramecin at 30°. The points on the curve represent the amount of activity left after  $\frac{1}{2}$  hour exposure to the particular pH.

as that described for *Paramecium aurelia* 51, variety 4. The solution was concentrated by filtration till about 5000 animals were present per cc. From this suspension approximately 0.5 cc. was added to depression slides. Ten depressions were used for a single determination. To each depression was added 0.1 cc. of the solution to be tested. The depression slides were stacked in glass moist chambers and incubated at 27°. After 48 hours, the dead and affected animals in each depression slide were counted by removal with a micro suction pipette. The number of animals killed in ten depres-



sions represents the activity of 1 cc. of the tested dilution. A wide variation in the activity of the untreated paramecin paste was always encountered. For this reason a control at pH 7.0 was included in every determination. In order to compare the results the actual counts were recalculated on a percentage basis. Where possible, the data were analyzed statistically according to the methods of Fisher (20).

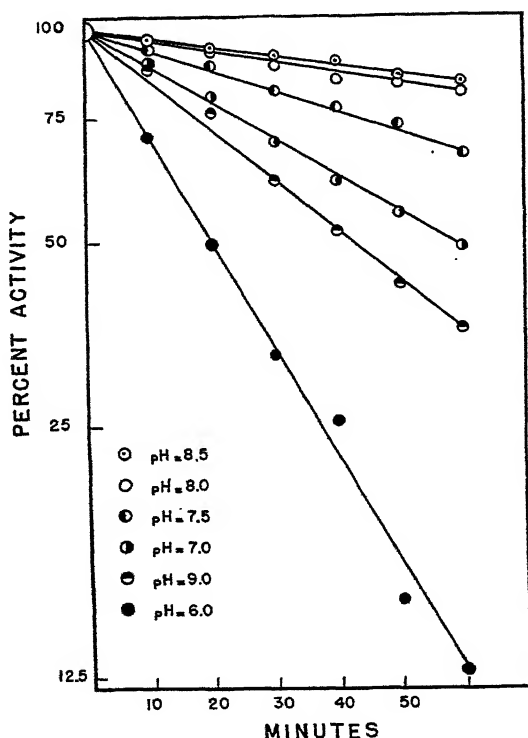


FIG. 3. The inactivation of crude paramecin preparations at different hydrogen ion concentrations at 30°. The inactivation is expressed as per cent activity left at each time interval.

### Results

The experiments and the conclusions derived from them are valid only if there exists a direct proportional relationship between the dilution of the solution containing the killer principle and the number of animals killed. Austin (3) and Sonneborn and coworkers (4) have presented evidence that the number of sensitive animals killed is directly proportional to the concentration of paramecin. Their experiments, moreover, have established the

fact that one single particle of paramecin can kill a sensitive *Paramecium*, thereby permitting computation of the concentration of paramecin in the solutions. We have again confirmed the direct proportionality between the concentration of paramecin and the number of sensitive animals killed under the conditions of our experiments. These results are given in Fig. 1. The number of sensitive animals killed is directly proportional to the dilution of the three reported preparations which differ greatly in their paramecin content.

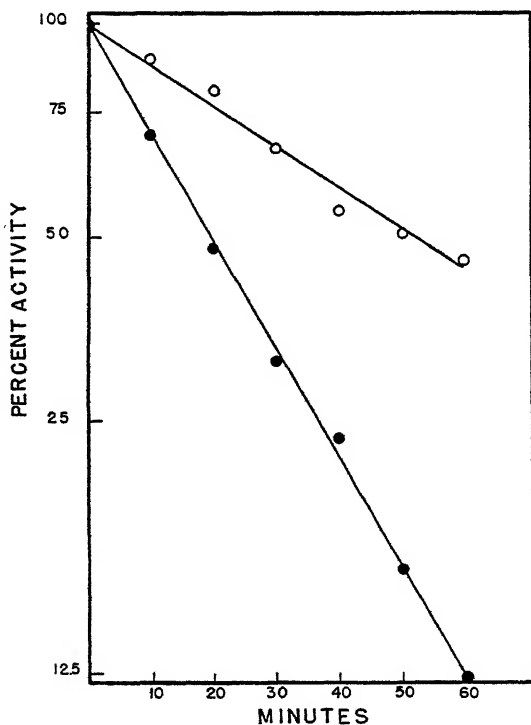


FIG. 4. The inactivation of crude paramecin preparations at 40°, expressed as per cent activity left at each time interval. ●, pH 7.0, ○, pH 8.0.

It is clear from Table I and Fig. 2 that paramecin (in the crude extract) has a narrow pH stability range. Even at the pH where the compound appears to be most stable, about 15 per cent of its initial activity is lost by an exposure to 30° for 1 hour. When the data of Table I are plotted in such a manner that  $\log C = kt$  (first order reaction), as is done in Fig. 3, it appears that the data fit a first order reaction curve very closely. It may be concluded here that the inactivation of paramecin at the different pH values is a first order reaction.

The inactivation of paramecin at 40° was investigated for only two hydrogen ion concentrations, pH 7.0 and pH 8.0. The mean values of three different determinations are represented in Fig. 4. The data also fit a straight line closely when  $\log_e C$  is plotted as a function of time. The reaction constant (first order reaction) at this temperature for the two hydrogen concentrations is pH 7,  $K_{40} = 0.0359$ ; pH 8,  $K_{40} = 0.0127$ . From the velocity of destruction at 30° and at 40° the activation energy for the inactivation of paramecin ( $\mu = \Delta H - RT$ ) has been calculated to correspond to 126,000 calories per mole at pH 7, and 169,000 calories per mole at pH 8, which are typical values for enzymes and proteins.

Unexpected results were obtained when the rate of inactivation of paramecin was investigated at 20°. These results are reported in Table II. There is an apparent increase in activity during the first 45 minutes

TABLE II  
*Inactivation of Crude Paramecin Preparation at pH 7 and pH 8 at 20°  
(Four Determinations)*

pH	Per cent activity						
	0 min.	10 min.	20 min.	30 min.	40 min.	50 min.	60 min.
7	100	131.5	144.7*	142.0	155.9*	142.5*	148.7
8	100	101.9	157.7*	128.6	129.2*	137.3*	131.8
	90 min.	120 min.	150 min.	180 min.	240 min.	300 min.	
7	131.3	127.4	120.0	118.2	100.8	93.0	
8	112.6	124.9		128.8	117.1	113.4	

\* Two determinations only.

Thereafter the activity drops slowly and attains a logarithmic rate after about 120 minutes. At present no explanation can be given for this phenomenon. It is conceivable that the observed increase in activity is due to a release or dissociation of paramecin from the bound state in which it is present in the cell. The inactivation proceeds simultaneously. After 45 minutes this process becomes the determining reaction.

#### DISCUSSION

It is evident from the experiments reported here that paramecin is an unstable compound. It may be concluded from the experiments that the inactivation at different hydrogen ion concentrations can be represented by a first order reaction. This is comparable to the results obtained by Chick and Martin (21), and those reported by Lauffer and Price (22). The former showed that the heat denaturation reactions of hemoglobin

and of egg albumin are first order reactions, while the latter showed that this was also the case for the thermal denaturation of tobacco mosaic virus. The average value for the activation energy for the inactivation of thirty-four different enzymes ( $\mu = \Delta H - RT$ ) was found to be  $\mu = 68,600$  calories per mole (23). The values have a range from 22,000 calories per mole to 198,000 calories per mole. Recent work (24) also indicates that the energies of activation are similar for enzyme inactivation and protein denaturation. The experimentally obtained values for the energy of activation for the inactivation of paramecin ( $\mu = 126,000$  calories per mole at pH 7, and  $\mu = 169,000$  calories per mole at pH 8) are within the limits for those reported for enzymes and proteins. It may be concluded then that paramecin belongs to either of the two classes, or both. The inactivation reaction must proceed at a much higher speed than the release of paramecin from the cell material, for it is surprising indeed that no evidence for a release of paramecin could be found at 30° or at 40°. It is possible that the release of paramecin is due to an enzymatic mechanism with a very low temperature optimum. It should, *a priori*, be possible to find an inhibitor for the inactivation reaction, thereby opening a way for obtaining a relatively richer source of paramecin. Investigations pertaining to this problem are in progress and will be reported at a later date.

#### SUMMARY

Paramecin, the killer substance of *Paramecium aurelia* 51, variety 4; is instantaneously inactivated at hydrogen ion concentrations ranging from pH 1.0 to 5.0 and pH 9.5 to 11.0. It is moderately stable in the pH range 7.0 to 9.0. Even in this range inactivation proceeds rapidly at the temperatures investigated. The activation energy for the inactivation of paramecin has the typical value for that of an enzyme or protein.

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# FIXATION OF ISOTOPIC NITROGEN BY EXCISED NODULES\*

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After 60 years of research in symbiotic nitrogen fixation, the agent of fixation has not been established. Apparently only two biological species, leguminous plants and *Rhizobium*, are essential (1), but attempts to separate the components have been unsuccessful. Unequivocal fixation by the intact nodule excised from a plant actively fixing nitrogen has not been demonstrated. Ordinarily, cells of plant and animal show no such sensitivity to the environment but will readily carry out their characteristic biological reactions in tissue culture. An explanation of this notable exception may be important for an understanding of the mechanism of the reaction.

Two possibilities appear worthy of investigation. (a) In the intact plant, the products of fixation are removed as rapidly as formed, but in the excised nodules, these accumulate and stop the reaction. If so, a very sensitive method for detection of fixation might be necessary. The usual Kjeldahl procedure with its attendant difficulties in sampling would not be suitable, but the much more sensitive isotopic method (2) might suffice. (b) An intermediate essential for fixation is supplied by the host plant. This postulated intermediate apparently is either present in a very low concentration or is so labile that it can disappear by the time  $N^{15}$  is supplied to the excised nodules.

Our initial experiments (2, 3) were inconclusive in that occasionally fixation was obtained, but its occurrence was not associated with any specific treatment, including supplying postulated intermediates, such as members of the tricarboxylic acid cycle. Recently, we have reexamined this problem in an effort to secure consistent fixation, or, failing this, to establish the origin of our occasional positive results.

## EXPERIMENTAL

Nodules from Canada field pea, soy bean, or cow-pea plants were picked directly into ice water, then transferred to culture vessels. 40 per cent po-

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tassium hydroxide was placed in the center well. The culture vessels were attached to a six place manifold, evacuated, and supplied 0.4 of an atmosphere of 50 per cent oxygen and 50 per cent nitrogen enriched with  $N^{15}$ . A 2 liter bulb, previously filled with oxygen at the pressure anticipated for the main system was opened to the manifold to replace the oxygen used for respiration. The vessels were immersed in a water bath at  $25^{\circ}$ . After 24 hours of incubation the contents of the flask were digested with sulfuric acid. The ammonia was distilled and converted with alkaline hypobromite to molecular nitrogen which was collected in a small gas bulb that fitted to the mass spectrometer. Details are provided by Burris *et al.* (2).

Experimental variations were made in the procedure to test the two hypotheses cited. To some of the culture vessels, 10 mg. of oxalacetic acid,  $\alpha$ -ketoglutaric acid, citric acid, or succinic acid were added as possible essential intermediates. On the assumption that excised leaves and stems may contain necessary photosynthetic products, these were added to some of the vessels.

To maintain the concentration of labile intermediates in the nodules, we usually picked these directly into ice water or froze them in liquid air. If frozen, all of the manipulations necessary to place the nodules in an atmosphere containing excess  $N^{15}$  could be completed before thawing occurred. An effort was made to increase the concentration of the postulated intermediates in the nodule by growing clover plants in the presence of molecular hydrogen or carbon monoxide. Although these two gases inhibit fixation, the nodules on the plants are large, malformed, and deep red in color. On transfer of such plants to a normal atmosphere, fixation begins immediately at an accelerated rate, possibly because of the accumulation of essential metabolites.

All experimental procedures except raising the plants were bacteriologically controlled; in one series, nodules from peas grown aseptically as described by Virtanen *et al.* (4) were tested.

#### DISCUSSION

Typical results of trials in which oxalacetic,  $\alpha$ -ketoglutaric, and citric acids were added to nodules are summarized in Table I. These data provide little basis for claims of fixation by excised nodules. Occasionally the gain in  $N^{15}$  approaches a statistically significant value (2), but these are border line instances. In many of these experiments, nodules were tested after addition of succinate as well as in the absence of any added metabolite. No fixation was observed. Also, the presence of excised stems and leaves would not induce fixation. Table II summarizes the data from three experiments in which abnormally large nodules were obtained from plants in which the fixation reaction had been inhibited.

The value of 0.08 atom per cent excess  $N^{15}$  observed in one instance alone approaches a significant gain.

TABLE I  
*Test of Fixation of  $N^{15}$  by Excised Nodules*

The values are expressed as atom per cent  $N^{15}$  excess over air controls.

Source of nodules	Treatment	Initial atmosphere	Substrate added		
			Oxalacetate	$\alpha$ -Keto-glutarate	Citrate
Peas	Chilled	20.28	-0.001	-0.002	-0.029
"	"	9.89	-0.022	-0.010	-0.007
"	aseptic	13.04	-0.005	0.003	0.022
"	Frozen in liquid air	9.89	-0.013		-0.023
"	" " " " " aseptic	4.49		-0.005	-0.004
"	" " " " " aseptic	13.04	0.086	0.061	-0.031
Soy beans	Chilled	14.90	-0.005	-0.010	0.022
"	"	21.62	-0.003	-0.001	
"	"	30.38	0.004	-0.014	0.005
" "	Frozen in liquid air	13.87	0.102	0.053	0.083
"	" " " " "	13.77	0.066	0.070	0.212
Cow-peas	Chilled	30.05	0.005	0.010	0.000
"	"	15.36	-0.015	0.000	-0.013
"	"	29.04	0.009	-0.003	0.026
"	Frozen in liquid air	10.97	0.000	-0.020	-0.026

TABLE II  
 *$N^{15}$  Fixation by Nodules and Rootlets from Red Clover*

The values are expressed as atom per cent  $N^{15}$  excess over air controls.

Red clover	Initial atmosphere	Plants grown in		
		Air	Air-CO	Air-H <sub>2</sub>
Nodules	9.90	0.000	-0.002	0.014
	4.53	0.017	0.030	0.083
	13.45	0.022	0.015	0.011
Rootlets	11.39	0.028	0.028	-0.019
	3.15		-0.015	-0.033
	4.53	-0.026	0.031	-0.005

In efforts to secure fixation during the past 5 years, we have tested 133 samples of nodules from plants grown under a variety of conditions and subjected to numerous diverse treatments. A summary of the results is given in Table III. If we adopt the statistically significant gain of 0.05 atom per cent excess as our criterion (2), twenty-one positive results have



been observed, or 16 per cent of the total. A more conservative criterion is about 0.1 atom per cent, as occasionally the mass spectrometer will be in error by 0.05 atom per cent. If this figure is adopted, the positive instances are reduced to ten, *i.e.*, 8 per cent of the total. Moreover three of these were obtained in a single experiment, which, because of the abnormally large gains observed, is at least open to suspicion (3).

From a detailed study of the results of all tests certain observations appear significant. (a) The positive results so far obtained are not consistently associated with any particular treatment and lend no support for the implication of any specific compound as an essential intermediate supplied by the plants. Relatively, the most consistent results have been obtained with nodules from plants in which the fixation process has been inhibited, but even here the positive findings are too few to be impressive. (b) The erratic occurrence of the positive results is reminiscent of the early

TABLE III

*Summary of Results on Fixation of  $N^{15}$  by Excised Nodules*

The values are expressed as atom per cent  $N^{15}$  excess over air controls.

Series No.	Samples tested	Gains of $N^{15}$				Bibliographic reference No.
		>0.05 atom per cent	Per cent of total	>0.1 atom per cent	Per cent of total	
I	34	5	14	3	9	(2)
II	24	7	29	5	21	(3)
III	75	9	12	2	3	This paper
	133	21	16	10	8	

experiments with the symbiotic nitrogen fixation system (1) and suggests that a similar explanation may be found, *i.e.* the rôle of accompanying bacteria is overlooked. Nearly all experiments made with excised nodules both isotopically and with other techniques have used non-sterile nodules. We have made a few with nodules from plants grown in so called aseptic cultures, but proof of the absence of contaminating bacteria would hardly satisfy a critical bacteriologist. Experimentally, when nodulated roots are used, more positive results are obtained, a result that may arise from better opportunity for the necessary contamination than from the presence of an essential intermediate in the root.

From these observations, we conclude that unequivocal demonstration of fixation by the excised nodule does not exist and that any future claims of fixation should be accompanied by adequate bacteriological proof of cultural control. This conclusion has a significant implication for the

mechanism of symbiotic nitrogen fixation, since it means that the agent of fixation is inferred rather than known with certainty. Isotopic studies have confirmed the generally accepted belief that the locale of fixation is the root system (5), presumably the nodules. Free-living cultures of *Rhizobium* will not assimilate molecular nitrogen, and it now appears that even so simple an operation as removal of the nodule from the host plant prevents fixation even though the organism is associated with the cells of the plant. Until this unexpected loss of function is explained, conclusions regarding the mechanism can at best be only tentative.

#### SUMMARY

Attempts to induce nodules excised from leguminous plants to fix molecular nitrogen by increasing the concentration of possible intermediates gave inconclusive results of the type previously observed.

From the results of 5 years experimentation with excised nodules by the sensitive isotopic technique, it is concluded that unequivocal evidence of fixation is lacking. As in nearly all of the experiments described in the literature nodules grown in a non-sterile environment have been tested, the inconsistent positive results reported may well have arisen from inadequate bacteriological control.

A consequence of this conclusion is that the agent responsible for fixation in the symbiotic system is not yet precisely defined.

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## EXCRETION IN REPTILES

### I. NON-PROTEIN NITROGEN CONSTITUENTS OF THE URINE OF THE SEA-TURTLE CHELONE MYDAS L.

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Very little is known about excretion in reptiles. Most of the earlier workers (1) occupied themselves with qualitative or approximate quantitative determination of some of the constituents of the urine. Among all these workers there is a general agreement that terrestrial reptiles, except the Chelonia, excrete mainly uric acid. On the other hand excretion in the Chelonia is in dispute. In the case of land tortoises, Magnus and Müller (2) working on *Testudo nigra*, Marchand (3) on *T. tabulata*, Schiff (4) on *T. tabulata*, Clementi (5) on *T. graeca*, claimed the presence of urea in the urine. Contrary to these authors, Mills (6), investigating the urine of *T. tabulata*, was unable to find urea and showed the presence of uric acid. Drilhon and Marcoux (7) found in the urine taken from the bladder of *T. mauritanica* a considerable amount of uric acid and purines in suspension and urea in solution. According to these authors almost all of the non-protein nitrogen of the clear urine was in the form of urea. In the case of aquatic turtles, Burian (8) working on *Thalassochelys* proved the presence of uric acid in the urine. Lewis (9) and Wiley and Lewis (10), working on *Chelone mydas* and *Chrysemys picta* respectively, found that urea constituted the greatest part of excretory nitrogen as compared to uric acid and ammonia.

In view of this controversy the present work was found necessary, especially since an investigation of some of the metabolic processes of reptiles had already been undertaken.

#### Material and Methods

Only four specimens of *Chelone mydas* were obtainable, two males, Animals A and B, two females, Animals C and D. They were all nearly of the same size and each weighed about 25 kilos. When the experiments were conducted (in March and April), the females were found to contain a large amount of eggs. The animals were decapitated and the blood collected in sterile bottles, containing neutral potassium oxalate (0.2 gm. per 100 cc. of blood). The animals were then opened, their bladders re-

moved, and the urine measured and kept in sterile bottles. The amount of urine collected was as follows: Animal A, 175 cc., Animal B, 110 cc., Animal C, 260 cc., and Animal D, 65 cc.

The pH of the urine, estimated immediately by the British Drug Houses capillator, was as follows: Animals A, B, C, and D, pH 4.4, 4.6, 4.4, and 8.5, respectively. The urine in all cases contained mucus and that of Animals A and C showed a slight deposit. The urine was cleared from mucus and deposit by centrifuging. Thymol was then added to the urine (1 mg. per cc. of urine) and the urine was kept in the ice chest. Under these conditions the pH remained unchanged.

A protein substance was found to be present in all specimens of urine. It was removed, when necessary, by heating the urine to boiling, acidifying with dilute acetic acid, and filtering.<sup>1</sup>

*Urine Analysis*—The total non-protein nitrogen was estimated by the Kjeldahl-Gunning method (11). Urea was estimated by the urease method of Van Slyke and Cullen (12) with acetate buffer, the urease extract being prepared according to the method of Damodaran and Sivaramakrishnan (13, 14) from watermelon seeds. Ammonia was estimated both by Folin's (15) and the micro-Kjeldahl method (16). Uric acid was estimated by the direct colorimetric procedure of Benedict and Franke (17) as modified by Christman and Ravwitch (18). Amino acids were estimated by Folin's colorimetric method (15). The high concentration of ammonia in urine necessitated the addition of 3 gm. of permutit for each 10 cc. of 1:20 urine, and the gentle agitation was prolonged for 15 minutes. This process was repeated twice. Urine after such treatment contained no traces of ammonia. Creatinine was estimated by Shaffer's colorimetric method (19); creatine by Folin's microchemical method (20). Griffith's method (21) was used for estimation of hippuric acid, and Larson's (22) for estimation of allantoin.

The deposits present in the urine of Animals A and C were examined microscopically and were found to consist of yellow uric acid crystals. These deposits were washed with warm water and then dissolved in a 2 per cent solution of piperazine and estimated colorimetrically as mentioned above.

*Blood Analysis*—Protein-free blood filtrate was obtained from unlaked blood by Folin's method (23). The total non-protein nitrogen in the

<sup>1</sup> The presence of such a protein substance was detected not only in these turtles but also in a number of other reptiles already examined by the author. The presence of this protein substance seems to have been overlooked by all earlier workers on the *Chelonia* except Mills (6) who demonstrated its presence in all of the animals he examined. The origin and significance of this protein substance are being investigated.

blood filtrate was estimated by the Kjeldahl-Gunning procedure (11). Ammonia was estimated according to Nash and Benedict (24). Urea was determined in the blood filtrate by urease hydrolysis, with acetate buffer, aeration, and nesslerization (15). Uric acid was estimated by Folin's direct method (25, 26). Amino acids were determined by Folin's procedure (27) as modified by Danielson (28) and Russell (29).

## RESULTS AND DISCUSSION

The results of the analysis of urine are shown in Table I. Ammonia forms the major excretory nitrogen end-product. This is in harmony with what is known in aquatic invertebrates and marine teleostian fishes (30-

TABLE I  
*Non-Protein Nitrogen Constituents of Urine of Chelone mydas*

Constituents	Nitrogen in 100 cc. urine					Nitrogen partition, per cent of total N excreted				
	Animal A, ♂	Animal B, ♂	Animal C, ♀	Animal D, ♀	Average, Animals A, B, C	Animal A, ♂	Animal B, ♂	Animal C, ♀	Animal D, ♀	Average, Animals A, B, C
	mg.	mg.	mg.	mg.	mg.					
Total N	288.7	399	262.5	210	316.7	100	100	100	100	100
Ammonia N	93.8	201.6	120.4	61.6	138.9	32.49	50.52	45.87	29.33	42.96
Urea N	None	None	None	24.7	None	None	None	None	11.75	None
Uric acid N	4.2	7.5	8.8	13.2	6.8	1.45	1.88	3.36	6.27	2.23
Allantoin N	12.6	47.9	64.4	15.6	41.6	4.37	12.0	24.53	7.44	13.63
Amino acid N	15.7	29.3	25.8	38.6	23.6	5.42	7.34	9.83	18.38	7.53
Hippuric acid N	66.5	42.0	21	31.5	43.2	23.04	10.52	8.0	15.0	13.85
Creatinine N	3.3	6.4	4.9	3.5	4.8	1.14	1.60	1.87	1.67	1.54
Creatine N	27.8	17.4	10.8	8.8	18.6	9.63	4.36	4.12	4.18	6.04
Undetermined N	64.8	46.9	6.4	12.5	39.3	22.4	11.8	2.4	6.0	12.2

32). Ammonia is also present in the blood (Table II). The mode of appearance of ammonia in the urine seems to be quite different from what is known to occur in mammals, in which ammonia is absent from the blood (33) and forms a low proportion of the excretory nitrogen in the urine. In this latter case, its formation and quantity are conditioned by the reaction of the urine. It is formed almost entirely in the kidneys (34-36), mainly from glutamine (37) and also from amino acids but not from urea (38, 39). As to the sea-turtle, although the study of the formation of ammonia is not complete, the data so far available seem to throw some light on this problem. From Table II it is seen that urea is present in the blood of all specimens examined, while Table I shows that it is absent from the urine of Animals A, B, and C and present only in the urine of Animal D (Table I).

The percentage of excreted ammonia nitrogen in Animal D is decidedly lower than the average for the other three animals. On the other hand the sum of the percentages of ammonia nitrogen and urea nitrogen of this animal comes very near to the average percentage of ammonia nitrogen for the other three animals. This, added to the fact that the blood of all the specimens examined contains an appreciable amount of ammonia as well as urea, suggests that the ammonia in the urine of the sea-turtle might originate partly from blood ammonia and partly from blood urea. This suggestion receives further support from the fact that the higher percentage of excretory ammonia in Animals A, B, and C is not accompanied with

TABLE II  
*Some Non-Protein Nitrogen Constituents of Blood of Chelone mydas*

Constituents	Nitrogen in 100 cc. blood			
	Animal A	Animal B	Animal C	Animal D
	mg.	mg.	mg.	mg.
Total non-protein N.....	108.7	560	280	420
Ammonia N.....	11.2	3.8	6.9	3.8
Urea N.....	25.5	40.4	16.7	14.2
Uric acid N.....	6.0	1.6	2.1	1.6
Amino acid N.....	15.9	9.2	15.0	9.8

TABLE III  
*Uric Acid As Deposit in Urine*

Animal	Total uric acid	Uric acid N	Uric acid N in 100 cc. urine
	mg.	mg.	mg.
A	45.5	15.17	8.67
C	79.8	26.6	10.23

decreased amounts of excretory amino acids. The part of ammonia which originates from blood urea is probably formed in the kidneys.

Table I also shows that the main part of the end-product of purine metabolism is in the form of allantoin and a small part in the form of uric acid. But it looks as if the ability of turtles to oxidize uric acid to allantoin is less than that of mammals, as the values of uricolytic index of these animals are as follows: Animal A 75, Animal B 86.5, Animal C 88, and Animal D 54.2. Moreover if the uric acid, which is found as a deposit in the urine of Animals A and C (see Table III) and which should be included with excretory uric acid, is taken into consideration, the values of the uricolytic index of these two animals will be 49.4 and 77.2 respectively.

In mammals the uricolytic index is much higher. The average for different rodents is 95, for different ungulates 90.2, and for different Carnivora 97 (40). It is remarkable that Wiley and Lewis (10), using the method of Christman, have failed to find allantoin in the urine of *Chrysemys picta*.

Table I shows also that hippuric acid is excreted in large amounts. This may be due to the herbivorous diet of these animals. Hippuric acid is already known to occur in large amounts in the urine of herbivorous mammals: horses (41), cows, oxen, and sheep (42).

The comparatively high concentrations of hippuric acid and creatine excreted by the animals investigated recalls what is known to occur in mammals (43, 44). Details of the mode of formation of these substances in sea-turtles are at present being investigated.

#### SUMMARY

1. Ammonia forms the major nitrogen end-product in the urine of *Chelone mydas*.
2. Urea is absent from the urine of the majority of the animals examined.
3. Allantoin forms the main end-product of purine metabolism. The uricolytic index is, however, less than that in mammals.
4. Hippuric acid is found in the urine in appreciable amounts.

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## THE ESTIMATION OF SERUM VITAMIN A WITH ACTIVATED GLYCEROL DICHLOROHYDRIN\*

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This paper deals with a new method for the estimation of vitamin A in blood serum based on the new colorimetric reaction between vitamin A and activated glycerol dichlorohydrin (GDH).

Most of the available methods for estimating vitamin A in blood serum depend upon the antimony trichloride reaction (1-6). The drawbacks to this reaction are that (1) the color is unstable and must be read within 5 seconds after addition of the reagent (unless low temperature is employed); (2) it is sensitive to moisture in air and reagents, and thus extreme anhydrous precautions must be observed; and (3) the reagent is corrosive and is a source of danger to the instruments employed.

During the course of our investigations Bessey *et al.* (7) reported a method for the determination of vitamin A in small quantities of serum which is dependent on the measurement of the ultraviolet absorption of serum extracts at 328  $m\mu$  before and after irradiation with ultraviolet light. These determinations require expensive equipment, including an ultraviolet spectrophotometer and an ultraviolet lamp for irradiation. Hemolyzed blood has been found to give higher carotene values, and due to the high blanks after irradiation, the results tend to be unreliable (see Table I).

Recently a new colorimetric reaction of vitamin A was reported, which takes place on the addition of practical (8) or activated (9) glycerol dichlorohydrin to a solution of vitamin A in chloroform. This new reaction appears to be suitable for quantitative purposes, since it obeys Beer's law over a reasonable range, and offers the following advantages over the widely used Carr-Price reaction (1): (1) the violet color produced is stable for from 2 to 10 minutes after the addition of the reagent, (2) the reagent is not affected by traces of moisture on the most humid days, (3) no film of antimony oxychloride is left on the cuvettes, (4) the reagent is practically non-corrosive, and (5) the stable color makes possible the use of a slow reading, null-point spectrophotometer (Beckman) in the measurement of the absorption of the violet color, (6) the interference of vitamins D<sub>2</sub> and

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D<sub>3</sub>, ergosterol, 7-dehydrocholesterol, and cholesterol upon the GDH reaction has been found to be negligible (8, 9).

In the application of GDH to vitamin A determinations on fish oils (10), the results in analyses with the reagent showed good agreement with analyses in which the SbCl<sub>3</sub> reaction is employed.

TABLE I

*Vitamin A and Carotene Values of Serum in Presence and Absence of Hemolyzed Red Blood Cells*

Hemolyzed cells	Log $\frac{I_0}{I}$ , 328 m $\mu$		Vitamin A	Carotene
	Before irradiation	After irradiation		
			<i><math>\gamma</math> per cent</i>	<i><math>\gamma</math> per cent</i>
Absent*.....	0.163	0.088	44	179
Present.....	0.315	0.200	67	220
Absent*.....	0.134	0.045	52	92
Present.....	0.645	0.515	76	200
Absent†.....			39	245
Present.....			41	235
Absent†.....			54	170
Present.....			53	170
Absent†.....			28	100
Present.....			28	100

\* Ultraviolet absorption method (7).

† SbCl<sub>3</sub> method (6).

TABLE II

*Influence of Moisture on Vitamin A Analysis at 37° and 50 Per Cent Relative Humidity*

The results are expressed as optical density, log  $I_0/I_t$ .

	CHCl <sub>3</sub>	SbCl <sub>3</sub>	GDH
Dry.....		0.1382	0.1904
Saturated with water.		0.1838 (Cloudy)	0.1904

Further studies of reagent properties have shown that in using vitamin A standards in chloroform which have been saturated with water at 37° and in an atmosphere of 50 per cent relative humidity the readings obtained in vitamin A determinations are identical with those with anhydrous standards when GDH is used as the colorimetric reagent. Under these same conditions the readings varied considerably between anhydrous and water-saturated standards when the Carr-Price reaction was used, as is shown in Table II. Attempts to read at 720 m $\mu$  the cloudiness interference encountered in the use of the SbCl<sub>3</sub> reagent under humidified conditions were never completely successful.

Cloudiness interference was also present and often uncontrollable in the use of the Carr-Price reagent when analyses were attempted on saponified human sera (7) and on whole rat sera. Neither of these conditions disturbed vitamin A analysis when GDH was the colorimetric reagent.

The reagent has also shown itself to give a relatively stable  $L_{1\text{ cm.}}^{1\%}$  value over a period of from 6 to 13 months, as is illustrated in Table III. The GDH checked was selected at random from batches of the reagent which had been activated in this laboratory. There were no special precautions taken in storage. The reagent had been kept on the shelf, subject to exposure to diffuse light, in clear, glass-stoppered Pyrex bottles.

The used reagent may be recovered and redistilled with  $\text{SbCl}_3$  (as described below). The activated reagent prepared from waste glycerol dichlorohydrin has been found to have good stability and good activity (Table III).

TABLE III  
*Deterioration of GDH As Measured by  $L_{1\text{ cm.}}^{1\%}$  Value of Color Produced on Addition of Vitamin A*

GDH source	1st $L_{1\text{ cm.}}^{1\%}$ value	Elapsed time	2nd $L_{1\text{ cm.}}^{1\%}$	Total deterioration	Average deterioration
		mos.		per cent	per cent per mo.
Eastman Kodak.....	1144	6	1015	11	1.8
Shell.....	1090	13.5	893	18	1.3
Shohan.....	1338	6	1313	1.8	0.30
Waste GDH redistilled.....	1437	7	1398	2.7	0.39

Preliminary studies (11) on the application of GDH to the estimation of serum vitamin A disclosed certain disadvantages of the reagent which would have to be removed. The  $L_{1\text{ cm.}}^{1\%}$  value of the reagent is only about one-fourth that of the  $\text{SbCl}_3$  reagent. The interference of carotene was higher than with  $\text{SbCl}_3$  when absorption measurements were made with a wide band width of light such as is given by the Coleman spectrophotometer or filter photometers.

Owing to the stability of the GDH-vitamin A color it is possible to read the color in a horizontal absorption cell which provides a long (50 mm.) light path with a volume that in the usual test-tube type of cuvette has a 13 mm. light absorption path. This color can so be read more sensitively than the  $\text{SbCl}_3$  color, which requires the test-tube type of cuvette for rapid reading. Fig. 1 shows the cuvette used in these studies, with a 50 mm. length and of 2.8 ml. capacity.

A further change in instrumentation, the use of a 555  $m\mu$  filter instead of Filter PC-4, in conjunction with the Coleman universal spectrophotometer

had a 2-fold benefit. The  $L_{1\text{ cm.}}^{1\%}$  value<sup>1</sup> of the GDH reaction was increased about 25 per cent and so was raised to the equivalence of the  $E_{1\text{ cm.}}^{1\%}$  value.<sup>2</sup> The  $E_{1\text{ cm.}}^{1\%}$  values obtained in the use of the Beckman spectrophotometer and the  $L_{1\text{ cm.}}^{1\%}$  values found with the two filter systems in the Coleman



Fig. 1. Schematic diagram of horizontal absorption cells of length 50 mm. The capacity is approximately 2.8 ml. The diameter of the cylindrical cuvette is approximately 8 mm.

TABLE IV  
*Comparison of Extinction Coefficients of Vitamin A-GDH Reaction*

Activated GDH Batch No.	Instrument		
	Beckman $E_{1\text{ cm.}}^{1\%} \text{ 555 m}\mu$	Coleman universal $L_{1\text{ cm.}}^{1\%}$	
		Filter PC-4	555 m $\mu$ filter
1	1420	1165	1440
2	1490	1165	1450
3	1470	1250	1500
4	1490	1190	1480
5	1330	1100	1340
Mean.....	1440	1170	1440

spectrophotometer on different batches of GDH are shown in Table IV. By the use of the 555 m $\mu$  filter and the longer absorption path, the optical densities of the color produced with GDH were of the same magnitude or higher than the densities of the color produced with SbCl<sub>3</sub> on equal amounts of vitamin A. With the use of the 555 m $\mu$  filter the carotene interference

<sup>1</sup> The term  $L_{1\text{ cm.}}^{1\%}$ , analogous to  $E_{1\text{ cm.}}^{1\%}$ , introduced by Dann and Evelyn (12) for use in photoelectric colorimeters which employ a band width of light of about 30 to 40 m $\mu$ , refers in this paper to the extinction coefficient obtained with the use of filter photometers and spectrophotometers (Coleman) which give a wave band of light about 35 m $\mu$  wide.

<sup>2</sup> The term  $E_{1\text{ cm.}}^{1\%}$  is used for the extinction coefficient obtained with instruments in which monochromatic light is employed.

in the GDH reaction was only 10 to 20 per cent higher than the interference found with  $\text{SbCl}_3$  in human blood concentrations (represented by 1.0 to 3.0  $\gamma$  of carotene per ml.) as is illustrated in Table V.

It was shown in a previous report (11) that GDH determinations gave higher values than antimony trichloride on whole serum. (In that study neither the 555  $m\mu$  filter nor the horizontal cuvette was used.) Since that report Bessey *et al.* (7) have shown that higher results were obtained on saponified serum than on whole serum with the  $\text{SbCl}_3$  reagent. We decided to compare whole and saponified serum determinations for the Carr-Price reaction and for the GDH reaction. The data obtained for sixteen sera studied are presented in Table VI. Our results with  $\text{SbCl}_3$  agreed with the findings of Bessey *et al.* The values on saponified sera were significantly higher than those on whole sera (for  $\text{SbCl}_3$ ). Vitamin A values on GDH

TABLE V  
Comparison of Carotene Interference in  $\text{SbCl}_3$  and GDH Reactions

Carotene  $\gamma$ per ml.	Vitamin A equivalent of color		GDH - $\text{SbCl}_3$
	$\text{SbCl}_3$	GDH	Difference
	$\gamma$	$\gamma$	per cent.
1.0	0.104	0.124	+19
2.0	0.200	0.230	+15
3.0	0.295	0.338	+15
4.0	0.337	0.446	+32
5.0	0.383	0.540	+41
7.2	0.481	0.910	+89
14.4	0.871	1.840	+111

determinations of whole serum were again found to be higher than the  $\text{SbCl}_3$  results on whole serum.

There is, however, good comparison between the saponified serum estimated with  $\text{SbCl}_3$  and both the whole and saponified sera estimated with GDH. This tendency for GDH values on saponified and unsaponified samples to be closer than the corresponding  $\text{SbCl}_3$  values was indicated in previous studies on fish oil (10).

The discrepancy in values between whole and saponified serum vitamin A found in the use of antimony trichloride did not occur as markedly in the case of whole and saponified sera determined with GDH. In comparing the means of the whole and saponified serum values determined with GDH and  $\text{SbCl}_3$ , shown in Table VI, it appears that the  $\text{SbCl}_3$  saponified value agrees more closely with the GDH value for saponified serum than with that for whole serum. It was found, however, that the average deviation of the GDH saponified value ( $\pm 7.6 \gamma$ ) from the  $\text{SbCl}_3$

saponified value was greater than the average deviation of the result for GDH whole serum ( $\pm 6.1 \gamma$ ) from that of  $\text{SbCl}_3$  saponified whole serum. *This suggests that saponification is not necessary when GDH is used*

TABLE VI

*Comparison of Vitamin A Values on Whole and Saponified Sera with GDH and Antimony Trichloride As Colorimetric Reagents*

The values are expressed in micrograms per 100 ml. of serum.

Serum No.	Whole serum			Saponified serum		
	Vitamin A		Carotene	Vitamin A		Carotene
	$\text{SbCl}_3$	GDH		$\text{SbCl}_3$	GDH	
1	42.6	47.7	129	45.3	30.3	153
2	39.1	32.8	109	41.0	37.0	119
3	30.0	42.9	150	43.5	36.9	143
4	37.0	31.0	135	43.8	29.8	142
5	32.2	45.6	141	42.5	52.9	145
6	22.7	36.7	168	33.8	46.2	187
7	30.8	37.0	105	36.1	31.0	109
8	21.8	34.9	141	24.8	45.4	152
9	31.3	40.9	136	44.4	43.9	139
10	24.2	33.6	159	46.2	43.3	167
11	38.3	39.8	151	41.9	45.4	157
12	34.8	41.7	122	53.5	44.4	143
13	21.8	37.2	107	30.8	35.2	111
14	34.3	44.7	117	47.0	44.8	110
15	31.0	48.4	99	33.7	46.9	93
16	37.0	47.3	190	51.3	51.3	200
Mean.....	31.8	40.1	134	41.2	41.5	141
$P^*$ .....	10 <sup>-2</sup>		0.6	0.9		
s.d.†.....	$\pm 1.4$	$\pm 1.4$		$\pm 1.4$	$\pm 1.1$	

\*  $P$  represents the frequency with which the difference between two means may be due to chance alone. When  $P = 0.05$  or less, the difference between two means is considered statistically significant (13). Since in the present study each observation in one series corresponds to an observation in another series, the small sample, pair method of Fisher (13) was used.

† Standard deviation of duplicates from the mean  $\sqrt{S(d^2)/(n-1)}$ ;  $d$  = individual deviation from the mean of the duplicates.

as the colorimetric reagent in serum vitamin A determinations. Preliminary studies in this laboratory on sera of subjects following high vitamin A intake indicate, however, that when abnormally high values are encountered (e.g. in vitamin A tolerance tests) values found with GDH are definitely higher with saponification than without saponification. These data are

shown in Table VII. *For most valid results, especially when high values are expected, saponification of the serum before extraction is therefore recommended.*

In view of the favorable properties of GDH, we considered the desirability of eliminating anhydrous precautions (drying serum petroleum ether extract with anhydrous  $\text{Na}_2\text{SO}_4$ , redistilling chloroform, and keeping it over the dry  $\text{Na}_2\text{SO}_4$ ) and dispensing with the redistillation of the petroleum ether.

Comparison was made of vitamin A values on whole serum, with GDH, (1) with anhydrous precautions and with redistillation of the petroleum ether, (2) without anhydrous precautions, but with redistilled petroleum ether, and (3) without anhydrous precautions, and without redistilling the petroleum ether.

TABLE VII

*Vitamin A Values Found with Use of GDH and  $\text{SbCl}_3$  on Saponified and Whole Sera, and with Ultraviolet Absorption Method of Bessey et al. (7) after Administration of Large Amounts of Vitamin*

Subject No.	Hrs. after administration of 6000 U. S. P. units vitamin A per lb. body weight	Vitamin A per 100 ml. serum				
		Saponified serum			Whole serum	
		GDH	$\text{SbCl}_3$	Ultraviolet absorption (7)	GDH	$\text{SbCl}_3$
		$\gamma$	$\gamma$	$\gamma$	$\gamma$	$\gamma$
1	3	406	322	357	366	305
1	6	182	183	186	158	144
2	3	885	927		656	
2	6	458	458		184	
Mean...		483	473		341	

As is shown in Table VIII, vitamin A values obtained with the simplification of reagents and procedure are in good agreement with those found when the more elaborate technique, necessary in  $\text{SbCl}_3$  determinations, was used.

Vitamin A values with and without anhydrous precautions compared well. The standard deviation of duplicates from their respective means with anhydrous precautions was  $\pm 2.1 \gamma$  (3.6 per cent) and  $\pm 1.4 \gamma$  (2.5 per cent) without anhydrous precautions. These data revealed no objection to the deletion of anhydrous precautions from the method of blood analysis when GDH is used as the colorimetric reagent. Values found with the further simplification, namely the use of analytical reagent grade of petroleum ether instead of the redistilled reagent, agreed well with those found when the petroleum ether was redistilled before use. The standard



deviation of duplicates from their means,  $\pm 0.5 \gamma$  (0.9 per cent), indicates that this modification may also be recommended when GDH is used.

TABLE VIII

*Effect of Removal of Anhydrous Precautions and of Use of Redistilled Reagents in Determination of Vitamin A with GDH. Comparison Also of Vitamin A and Carotene Values with 450 m $\mu$  Absorption Method and 800 m $\mu$  GDH Method for Carotene Determination*

The values are expressed in micrograms per 100 ml. of serum.

Serum No.	Carotene determined at 440 m $\mu$			Carotene determined at 800 m $\mu$		
	(a)*	(b)*	(c)*	(a)	(b)	(c)
Vitamin A						
1	63.7	63.4	58.0	63.3	65.0	60.7
2	57.7	60.8	60.0	56.1	60.6	61.0
3	43.2	47.4	39.3	39.0	41.4	37.4
4	71.8	71.8	78.2	72.4	72.2	76.6
5	39.7	39.6	39.1	40.3	39.7	39.1
6	58.6	55.3	52.6	58.7	53.8	52.4
7	59.8	58.6	59.5	60.6	59.1	59.7
Mean.....	56.4	56.7	55.2	55.8	56.0	55.3
S.D.†.....	$\pm 2.1$	$\pm 1.4$	$\pm 0.5$	$\pm 1.4$	$\pm 0.5$	$\pm 0.5$
Carotene values						
1	221	225	230	226	208	205
2	160	164	171	176	166	166
3	150	142	150	192	198	165
4	139	131	139	134	128	151
5	100	96	91	94	94	91
6	155	143	131	154	158	133
7	127	131	127	119	127	125
Mean.....	150	147	148	156	154	148
S.D.†.....	$\pm 4.8$	$\pm 6.3$	$\pm 4.2$	$\pm 7.8$	$\pm 13.6$	$\pm 5.7$

\* (a) anhydrous precautions, redistilled petroleum ether; (b) no anhydrous precautions, but redistilled petroleum ether; (c) no anhydrous precautions, no redistilled petroleum ether.

† Standard deviation of duplicates from the mean. In statistical evaluation of the means there was no significant difference found between any of the means for both vitamin A and carotene values.

The absorption spectra for vitamin A and carotene reactions with GDH further suggested that carotene could be determined at 800 m $\mu$  from the same solution used to measure at 550 m $\mu$  the absorption due to vitamin A.

The separate step for carotene estimation necessary when  $\text{SbCl}_3$  reagent is employed could be eliminated. Instead of the quantitative transfers of the petroleum ether extract to and from the absorption cell for determination of carotene at  $440\text{ m}\mu$ , the extract could be directly evaporated and the dried extract taken up in chloroform and treated with GDH. The vitamin A reading would be taken at  $550\text{ m}\mu$  and the carotene reading at  $800\text{ m}\mu$  from the same solution.

Comparisons were made of carotene and vitamin A values found for whole sera between the  $440\text{ m}\mu$  carotene absorption and the  $800\text{ m}\mu$  GDH-carotene methods for carotene determination. As the data presented in Table VIII show, the values found for vitamin A and carotene in the final simplification of the method (no anhydrous precautions, no redistilled reagents, and carotene measurement at  $800\text{ m}\mu$ ) compare favorably with the values found with the method as it is conventionally used.

The measurement of carotene at  $800\text{ m}\mu$  has the disadvantage of a relatively low  $L_1^{1\%}$  value (9) for GDH-carotene at this wave-length. However, the use of instruments capable of transmitting light of wave-length  $830\text{ m}\mu$  should obviate this difficulty, as is indicated in the carotene-GDH absorption curve (9). Nevertheless, the measurement at  $800\text{ m}\mu$  entails only slightly less precision than at  $440\text{ m}\mu$ . The standard deviation of duplicates from their means for carotene when measured at  $440\text{ m}\mu$  was  $\pm 4.2\%$  (2.8 per cent) and  $\pm 5.7\%$  (3.8 per cent) when measured at  $800\text{ m}\mu$  with GDH (determinations in both cases without anhydrous precautions or redistilled reagents). Measurement of carotene-GDH color at  $830\text{ m}\mu$  being more sensitive, and so in an area of greater accuracy for the spectrophotometer, should make for greater precision in this method of carotene determination.

This difference in precision for carotene estimation between the two methods was not reflected in the eventual vitamin A precision. The standard deviation of duplicates for vitamin A when carotene was measured at  $440\text{ m}\mu$  was  $\pm 0.5\%$  (0.9 per cent) and also  $\pm 0.5\%$  (0.9 per cent) when carotene was determined at  $800\text{ m}\mu$  with GDH.

The simplification of vitamin A analysis with the use of GDH as the colorimetric reagent dispenses with the necessity of anhydrous precautions and redistilled reagents. For instruments capable of transmitting wave-lengths of light of  $800\text{ m}\mu$  or over, the measurement of carotene need not entail an entirely separate set of procedures, but can be obtained from the same GDH solution used for vitamin A estimation.

### *Method*

#### *Reagents—*

1. 95 per cent ethanol (U. S. P.).

2. 1 N KOH in 90 per cent ethanol freshly prepared (stock solution prepared from 1 part of a stock solution of 1 N KOH and 10 parts of absolute alcohol. The stock solution of KOH may be used for several months.)

3. Petroleum ether, b.p. 30–60°. (a) Analytical reagent grade; (b) analytical reagent grade, distilled; fraction between 30–60° used.

4. Nitrogen, Ohio Chemical Company.

5.  $\text{Na}_2\text{SO}_4$ , anhydrous, analytical reagent grade.

6. Chloroform. (a) Analytical reagent grade; (b) analytical reagent grade, washed with water, distilled, first and last fractions discarded, and kept over dry  $\text{NaSO}_4$ .

7. Antimony trichloride, analytical reagent grade.

8. Carr-Price reagent; 30 gm. of  $\text{SbCl}_3$  in 100 ml. of chloroform solution.

9. Carotene; 90 per cent  $\beta$ -, 10 per cent  $\alpha$ -, from General Biochemicals, Inc.

10. Standard vitamin A solution. About 40 to 50 mg. of a vitamin A concentrate, which comes in gelatin capsules, control PC-3 from Distillation Products, Inc., were diluted in chloroform to give the desired concentrations of vitamin A.

The concentrate has an  $E_{1\text{ cm}}^{1\%}$  value in absolute ethanol of 100.75. This value was supplied by the manufacturers and agreed with that found by the authors. Multiplying this value by 2000, the standard commercial conversion factor, permits estimation of the vitamin A potency at 201,500 U. S. P. units per gm. If the extinction of the concentrate is divided by the extinction of crystalline vitamin A alcohol in ethanol, 1780, the vitamin A content of the concentrate becomes by simple proportion 5.56 per cent.

11. Activated GDH. Glycerol dichlorohydrin (Eastman Kodak, practical grade; a mixture of 1,3- and 2,3-dichlorohydrin from the Shell Chemical Company) is vacuum-distilled in the presence of approximately 1 per cent by weight of  $\text{SbCl}_3$  at from 10 to 40 mm. of pressure, the first and last fractions being discarded. The distilled reagent should be colorless and clear (a sample of the reagent should not become even slightly cloudy on the addition of water). The presence of  $\text{SbCl}_3$  in the distillate (shown when a sample of GDH becomes cloudy upon the addition of water) can be remedied by a second vacuum distillation. (It is recommended that an oil bath plus a water pump be used in distillation.) The activated reagent may be obtained from the J. B. Shohan Laboratories, 78 Wheeler Point Road, Newark 5, New Jersey.

#### *Apparatus—*

1. Coleman universal spectrophotometer, model 11. The instrument was calibrated according to the directions given by the manufacturer. This instrument employs a band of light 35  $m\mu$  wide.

2. 1.3 cm. cuvettes and cuvette carrier obtained from the Coleman Electric Company.

3. 5.0 cm. cuvettes obtained from the Pyrocell Manufacturing Company, 207 East 84th Street, New York 28. Carrier obtained from the Coleman Electric Company.

4. 555  $m\mu$  filter (filter combination 3482, M608, 1.0 mm., and 5300, M954, 4.0 mm.) obtained from the Corning Glass Works, Corning, New York.

*Procedure for Vitamin A Analysis of Serum (with Coleman Spectrophotometer)*

1 ml. of serum is pipetted into a  $\frac{3}{8} \times 4$  inch test-tube. 1 ml. of 95 per cent ethanol is added and the contents of the tube mixed by tapping (or for saponification 1 ml. of the 1 N KOH in 90 per cent ethanol is added, and the contents of the tube mixed and placed in a 60° oven for 20 minutes). 2 ml. of analytical reagent petroleum ether are added, and the tube is shaken for 10 minutes. After shaking, the tube is centrifuged for about 30 seconds. The supernatant petroleum ether is aspirated and placed in a  $\frac{1}{2} \times 4$  inch test-tube. The aspirator is a fine tipped dropper. With another 2 ml. of the petroleum ether, and shaking for only 5 minutes, the extraction procedure is repeated. (If carotene is to be determined at 440  $m\mu$ , the petroleum ether extract is quantitatively transferred to a 13 mm. cuvette, and the volume brought to a 4.0 ml. graduation marked on the side of the cuvette. The absorption is read at 440  $m\mu$  with Filter PC-4. The extract is quantitatively transferred to a  $\frac{1}{2} \times 4$  inch test-tube.)

The extract is evaporated to dryness by placing the tube in a 40–50° water bath and running a stream of nitrogen over it. 1.0 ml. of analytical reagent grade chloroform is added to bring the dried extract into solution. 4 ml. of GDH are added. The chloroform solution and the GDH are mixed with a flat tipped stirring rod. 2 minutes after mixture the solution is placed in a 50 mm. cuvette. The absorption of the solution is measured first at 550  $m\mu$  (with the 555  $m\mu$  filter) against a blank consisting of 4 ml. of GDH and 1 ml. of chloroform.

The wave-length dial is then turned to 800  $m\mu$ , the 555  $m\mu$  filter replaced with Filter PC-5 and at 4 minutes after the initial mixture of the reagents the absorption is read at 800  $m\mu$ .

The absorption at 800  $m\mu$  will give the carotene content (from the carotene calibration chart). The carotene content enables the evaluation of the optical density at 550  $m\mu$  which is due to carotene (from the carotene interference chart). The optical density for carotene interference is subtracted from the total optical density at 550  $m\mu$ . The resultant optical density gives the vitamin A value (from the vitamin A calibration chart). (The GDH-chloroform solution when prepared as described above has a volume of 5.0 ml. Since the horizontal cuvette has a capacity of less than

3 ml., it is possible to perform the analysis on 0.6 ml. of serum by reducing the amount of all reagents used by six-tenths. The intensity of the color produced by the 3.0 ml. of GDH-chloroform solution prepared in this manner will be the equivalent of the 5.0 ml. of solution obtained when 1.0 ml. of serum is used for determinations.)

#### *Preparation of Calibration Charts*

*Vitamin A*—The vitamin A reference graph is prepared with standards in chloroform solution containing from 0.2 to 5.0  $\gamma$  of the vitamin per ml. The vitamin A-containing standard solution as well as the GDH is preheated to 25° before use. To 1.0 ml. of the standard in a glass-stoppered cylinder are added 4.0 ml. of GDH. The contents of the cylinder are mixed by inversion and are placed in a 25° water bath for 2 minutes. The solution is then poured into the 5.0 cm. cuvette (see Fig. 1). The absorption of the pink color produced is read against a blank consisting of 4.0 ml. of GDH and 1.0 ml. of chloroform. The instrument is used with the wave-length scale set at 550  $m\mu$  in conjunction with a 555  $m\mu$  filter. Optical density is plotted against vitamin A content (per ml.) of standard used. (The 1 ml. of chloroform, in which the vitamin A extract is dissolved, represents the vitamin A in 1 ml. of serum; the calibration chart can so be used to read directly the amount of vitamin A in 1 ml. of serum.)

*Carotene Determined at 440  $m\mu$* —The absorption of solutions of carotene in petroleum ether containing 1.0 to 10.0  $\gamma$  of carotene per 4 ml. is read against a petroleum ether blank. When optical density is plotted against carotene concentration (per 4 ml.) a straight line should result. (Since the carotene content of 1 ml. of serum is extracted with, and read from, 4 ml. of petroleum ether, the concentration per 4 ml. read from the calibration chart actually represents the concentration per 1 ml. of serum.)

*Carotene Determined with GDH at 800  $m\mu$* —The chart is prepared in the same manner as the vitamin A graph with use of chloroform solutions of carotene containing from 1.0 to 10.0  $\gamma$  of carotene per ml. (Absorption is read at 4 minutes after reagent mixture.) Optical density at 800  $m\mu$  is plotted against carotene concentration (per ml.). Filter PC-5 is used with the Coleman spectrophotometer. In instruments capable of transmitting light of wave-length 830  $m\mu$  this wave-length should be used for carotene determinations with GDH.

*Carotene Interference*—This graph is prepared in the same manner as that for vitamin A, carotene being substituted for vitamin A in concentrations of from 1 to 10  $\gamma$  per ml. (The reading is taken at 550  $m\mu$  with the 555  $m\mu$  filter.)

This graph is used to correct the vitamin A reading at 550  $m\mu$  for the

interference of carotene as follows: From the optical density at 440 or 800  $m\mu$  the carotene concentration is found (with the carotene graph). The optical density which this amount of carotene will produce at 550  $m\mu$  is found from the carotene interference graph. This optical density is subtracted from the total optical density found at 550  $m\mu$ . From the corrected 550  $m\mu$  optical density, the vitamin A concentration is found on the vitamin A graph.

#### *Application of Method to Other Instruments*

*Beckman Spectrophotometer*—The vitamin A is evaluated at 555  $m\mu$  and the carotene at 830  $m\mu$ . The residue from the petroleum ether extract of 1 ml. of serum is dissolved in 0.2 ml. of chloroform and is treated with 0.8 ml. of GDH. The optical densities are measured in absorption cells of 1.0 cm. light path and of 1.0 ml. capacity. For cells of smaller volume but with the same length of light path, the amount of serum may be reduced proportionately to the reduction of reaction mixture required, with corresponding reduction of the volumes of chloroform and GDH.

*Filter Photometers*—By employing 555  $m\mu$  and 440  $m\mu$  filters (or an 800  $m\mu$  filter) the method may be used on any of the filter photometers. The volume of serum may have to be increased, depending on the length of the light path and the volume of colored solution necessary, as shown below:

$$\text{Volume of serum required} = \frac{\text{Volume of } \text{Cl}_3\text{CH} + \text{GDH (1:4) mixture required}}{\text{Length of light path in cm.}}$$

*Visual Colorimeter*—For the visual colorimeter the procedure is the same except that the dried extract of 1 ml. of serum should be taken up in 0.2 ml. of chloroform instead of 1.0 ml., and 0.8 ml. of GDH is used instead of 4.0 ml. The colors of the unknown and of a standard are compared in micro cups having a 1 ml. capacity and of 40 mm. length. The standard is prepared from 1 ml. of a chloroform solution of vitamin A containing 2.5  $\gamma$  of the vitamin per ml. and 4.0 ml. of GDH. Each unknown requires an independent standard, simultaneously prepared.

#### EXPERIMENTAL

*Humidity Studies*—The study of the effect of humidity on the GDH reaction described in Table II was made in a 37° walk-in incubator. Pans of water were placed in the incubator for about 24 hours prior to the time the actual determinations were done in order to create a humid atmosphere. Relative humidity was measured from readings on whirled wet and dry bulb thermometers. Standards were prepared under anhydrous conditions in the laboratory. A portion of the vitamin A-chloroform solution was saturated with moisture by shaking with distilled water.

*Carotene interference* was determined by measuring directly absorption due to known amounts of carotene, and also by measuring the increase in optical density due to the addition of known amounts of carotene to known amounts of vitamin A. The interference in the  $\text{SbCl}_3$  reaction was measured at  $615\text{ m}\mu$  with Filter PC-4 and for the GDH reaction at  $550\text{ m}\mu$  with the  $555\text{ m}\mu$  filter on the Coleman universal spectrophotometer.

*Comparison of Whole and Saponified Sera*—In comparing whole and saponified sera with GDH and  $\text{SbCl}_3$  as colorimetric reagents, all determinations were made on 1.0 ml. samples of serum. Whole sera were treated with 1 ml. of 95 per cent ethanol, and for saponification the sera were treated with 1 ml. of 1 N KOH in 90 per cent ethanol and heated at  $60^\circ$  for 20 minutes (as recommended by Bessey *et al.* (7)).

For extraction, in all cases 2 ml. of the redistilled petroleum ether were added to the treated sera in  $\frac{3}{8} \times 4$  inch test-tubes. The test-tubes were sealed with size 00 rubber stoppers which had previously been extracted with petroleum ether. Shaking (in a shaking machine) for 10 minutes followed. The tubes were centrifuged at slow speed for only 30 seconds (to prevent packing of the precipitate). The supernatant petroleum ether, containing the extracted vitamin A, was aspirated into another  $\frac{3}{8} \times 4$  inch test-tube. A piece of glass tubing drawn to a capillary tip was fitted with a rubber bulb for this purpose. The aspirator was made long enough so that the extract would not come in contact with the rubber bulb. Another 2 ml. of petroleum ether were added to the once extracted serum and the extraction procedure repeated, this time with shaking for only 5 minutes.

The petroleum ether extract was dried by adding a small amount of the  $\text{Na}_2\text{SO}_4$  and allowing it to stand for about 20 minutes. The extract was then poured into a square 13 mm. cuvette and, with petroleum ether washing of the  $\text{Na}_2\text{SO}_4$  and rinsing of the test-tube, the extract was brought to a 4 ml. graduation marked on the ground glass surface of the cuvette. The absorption at  $440\text{ m}\mu$  against a petroleum ether blank was used to determine carotene content.

The extract was transferred again to a  $\frac{1}{2} \times 4$  inch test-tube, with petroleum ether rinsing of the cuvette. The extract was evaporated to dryness under a stream of nitrogen in a  $40\text{--}50^\circ$  water bath.

For  $\text{SbCl}_3$  readings, 0.5 ml. of the dry chloroform was added to the test-tubes to bring the dried extract into solution. The 0.5 ml. of chloroform was poured from the tube into the 13 mm. cuvette. Another 0.5 ml. of  $\text{CHCl}_3$  was added to the test-tube and again poured into the cuvette. To the solution in the cuvette were added 3 ml. of the  $\text{SbCl}_3$  reagent. Readings of the maximum "stable" deflection of the galvanometer were made within 5 seconds after the addition of the Carr-Price reagent at

615  $m\mu$  against a 1:3  $\text{CHCl}_3$ - $\text{SbCl}_3$  blank. 0.1 ml. of acetic anhydride was added to 10 ml. of  $\text{SbCl}_3$  reagent immediately before using.

For GDH the dried extract was brought to solution by the addition of 1.0 ml. of  $\text{CHCl}_3$  to the test-tube. 4 ml. of GDH were added to the chloroform solution. Complete mixture of the reagent and sample was achieved (by an up and down motion) with a flat tipped stirring rod.

The test-tube was placed in a 25° constant temperature bath. About 90 seconds after mixture the solution was poured into the 50 mm. cuvettes (Fig. 1). At 2 minutes after mixture the reading at 550  $m\mu$  was taken on the per cent transmission scale against a chloroform-GDH blank set at 100 per cent transmission. All readings were taken on the Coleman universal spectrophotometer. Filter PC-4 was used when carotene, at 440  $m\mu$ , and vitamin A, at 615  $m\mu$ , were determined. With GDH the 555  $m\mu$  filter was used at 550  $m\mu$ .

#### *Elimination of Anhydrous Precautions and Redistilled Reagent*

In studying the effect of removal of anhydrous precautions and of dispensing with the use of redistilled reagents the technique was modified only as to the type of reagent used and the elimination of the step of drying with anhydrous  $\text{Na}_2\text{SO}_4$ . In these series the readings at 550  $m\mu$  were taken by the null-point method. The blank was set at 0 on the galvanometer scale and at 100 on the drum dial.

For carotene determinations at 800  $m\mu$  the GDH- $\text{CHCl}_3$  solution was kept in the cuvettes after reading at 550  $m\mu$ . The wave-length dial was changed to 800  $m\mu$ , and the 555  $m\mu$  filter replaced by the Coleman Filter PC-5. The drum dial was set at 100 per cent  $T$  and the galvanometer scale reset at 0 for the blank. The readings were taken at 800  $m\mu$  about 4 minutes after mixture of the GDH and chloroform solution. (The cuvettes were rinsed with chloroform between determinations and dried.)

Correction for the presence of carotene was made essentially by the method of Dann and Evelyn (12) in the serum determinations.

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#### SUMMARY

A method is described for measuring carotene and vitamin A in 1 (to 0.6) ml. of serum with activated glycerol dichlorohydrin (GDH) as a colorimetric reagent.

Changes in instrumentation (the use of a 50 mm. horizontal cuvette and a 555  $m\mu$  filter) have made the GDH measurement of vitamin A as sensi-



tive as the Carr-Price reaction. Carotene interference with the suggested filter is found to be only 10 to 20 per cent higher with GDH than with  $\text{SbCl}_3$  in the carotene concentrations found in blood.

Study of reagent properties showing non-interference of moisture in the reaction is described.

The method has been compared with the Carr-Price method on whole and saponified sera. Values with GDH on whole and saponified sera and values with  $\text{SbCl}_3$  on saponified sera compare favorably. Whole sera estimated with  $\text{SbCl}_3$  gave low values.

Simplifications of procedure found possible with the use of GDH are (1) elimination of anhydrous precautions, (2) elimination of redistilled reagents, and (3) the measurement of carotene at 800  $\text{m}\mu$  from the same GDH solution that is used for vitamin A estimation.

The application of the method to use on the Coleman spectrophotometer, the Beckman spectrophotometer, the filter photometer (electric colorimeter), and the visual colorimeter is described.

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## FATTY ACID METABOLISM

### VI. CONVERSION OF ACETOACETATE TO CITRATE IN ANIMAL TISSUES, STUDIED WITH ISOTOPIC CARBON\*

By NORMAN F. FLOYD, GRACE MEDES, AND SIDNEY WEINHOUSE

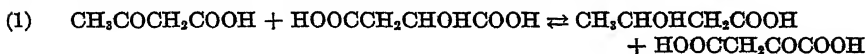
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Pennsylvania, Marcus Hook)*

(Received for publication, August 21, 1947)

In Paper V of this series (1) it was found that the citrate which accumulates during the oxidation of  $C^{13}$ -tagged acetoacetate by rat kidney in the presence of oxalacetate had a high content of isotopic carbon, thus confirming the participation of tricarboxylic acids in the oxidation of fatty acid intermediates. The present study represents a continuation of the investigation of this reaction with the objects of determining (a) to what extent acetoacetate contributes to citrate formation under anaerobic conditions, (b) whether acetoacetate is converted to citrate in other tissues besides kidney (2), and (c) to what extent the appearance of isotopic carbon in the citrate could be attributed to assimilation of  $CO_2$ .

#### *Results*

*Anaerobic Formation of Citrate from Isotopic Acetoacetate*—Krebs and Eggleston (3) found that the acetoacetate which disappears anaerobically in sheep heart in the presence of oxalacetate could be quantitatively accounted for as  $\beta$ -hydroxybutyrate, from which they concluded that the small amount of citric acid formed under these circumstances came exclusively from oxalacetate, the effect of acetoacetate being merely to maintain the concentration of oxalacetate by means of the following equilibrium.



From our previous results (1), however, it appeared very likely that acetoacetate carbon contributes to citrate formation also under anaerobic conditions. This proved to be true, as is shown by the results of the first experiment recorded in Table I. As in the previous aerobic experiments, rat kidney homogenate, 90 gm., was incubated in a saline solution contain-

\* With the technical assistance of Mary Cammaroti, Ruth Millington, and Ethel Niessen.

The authors wish to express their appreciation to the Sun Oil Company, Philadelphia, for their generous assistance in this work.

ing 0.01 M acetoacetate, 0.02 M oxalacetate, and 0.03 M  $\text{Ba}^{++}$  ions, the only difference being substitution of oxygen by nitrogen as the gas phase. A total of 54 mg. of citric acid was formed having a  $\text{C}^{13}$  excess of 0.78 per cent. Degradation by the procedure described previously (1) revealed the presence of 2.22 per cent  $\text{C}^{13}$  in the primary carboxyls, 0.20 per cent in the tertiary  $\text{COOH}$ , and none in the non- $\text{COOH}$  carbons. The absence of any

TABLE I

$\text{C}^{13}$  Distribution in Products of Oxidation of  $\text{C}^{13}$ -Tagged Acetoacetate in Presence of  $\text{Ba}^{++}$  Ions and Oxalacetate

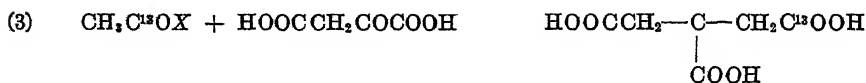
2 hours at  $38^\circ$

	Tissue					
	Kidney, 90.0 gm.; anaerobic		Muscle, 100 gm.; aerobic		Brain, 38.3 gm.; aerobic	
		$\text{C}^{13}$ excess		$\text{C}^{13}$ excess		$\text{C}^{13}$ excess
	mm	per cent	mm	per cent	mm	per cent
Acetoacetate, start.....	2.87	3.83	3.68	3.50	1.41	3.10
“ end.....	0.72	3.78	2.44	3.45	0.62	3.06
“ used.....	2.15		1.24		0.79	
Respiratory $\text{CO}_2$ .....		0.40		0.75		0.61
Citric acid.....	0.28	0.78	0.42	0.49	0.35	0.26
Primary $\text{COOH}$ .....		2.22		1.30		0.73
Tertiary $\text{COOH}$ .....		0.20		0.25		0.21
Non- $\text{COOH}$ carbons.....		0.00		0.00		0.00

appreciable dilution of the isotopic acetoacetate indicates that there was no endogenous acetoacetate formation.



OH



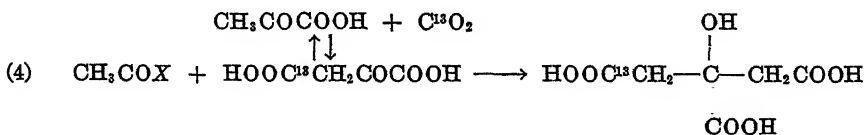
On the assumption that one of the two citrate primary carboxyls is derived from either a  $\beta$  or a carboxyl carbon of acetoacetate according to equations (2) and (3), we can calculate that of the total citrate formed,  $2.22 \times 100/3.83 = 58$  per cent, came from acetoacetate carbon.

*Citrate Formation from Acetoacetate in Muscle and Brain*—Breusch's observation that citrate is formed by a variety of animal tissues in the presence of acetoacetate and oxalacetate (notably muscle, heart, kidney, brain (2)) constituted the first suggestion of the wide scope of the tri-carboxylic acid cycle in acetoacetate oxidation, but the possibility remained

that in certain of these tissues oxalacetate or endogenous substrates other than acetoacetate served as the sole source of carbon for citrate formation. This might be expected, particularly in such tissues as muscle and brain, in which carbohydrate is considered to be the sole or predominant substrate undergoing oxidation. However, as is shown in the second and third experiments of Table I, the citrate formed during the oxidation of isotopic acetoacetate by rat skeletal muscle and brain had a high  $C^{13}$  content and hence was derived in substantial part from acetoacetate. With muscle the citrate had a  $C^{13}$  excess of 0.49 per cent, distributed as follows: 1.30 in the primary carboxyls, 0.25 in the tertiary  $COOH$ , and none in the non- $COOH$  carbons. Calculation indicates that in this tissue  $1.30 \times 100/3.50 = 37$  per cent of the total citrate was derived by equations (2) and (3) from acetoacetate carbon. With brain homogenate the citrate had a  $C^{13}$  excess of 0.26 per cent, distributed as follows: 0.73 per cent in the primary carboxyls, 0.21 in the tertiary  $COOH$ , and none in the non- $COOH$  carbons. The percentage of the total citrate derived from acetoacetate by equations (2) and (3) is thus  $0.73 \times 100/3.10 = 24$ . In neither muscle nor brain was there any significant dilution of the acetoacetate, denoting the absence of endogenous acetoacetate formation.

*CO<sub>2</sub> Assimilation in Citrate Formation*—In all three of the experiments of Table I, as well as in the previous ones (1), the  $C^{13}$  content of the primary carboxyl positions was higher than the respiratory  $CO_2$ ; hence, the latter could be excluded as the sole source of the isotopic carbon in this position of the citrate molecule. It was possible, however, and indeed likely, that part of the  $C^{13}$  appearing in this position, as well as the  $C^{13}$  present in small amount in the tertiary carboxyl, represents the assimilation of isotopic  $CO_2$  resulting from acetoacetate oxidation. Accordingly, three aerobic experiments were carried out with kidney, muscle, and brain, in which non-isotopic acetoacetate was oxidized in a medium of 0.02 M bicarbonate, having an 8 per cent  $C^{13}$  excess. The results of these experiments, recorded in Table II, reveal that assimilation of bicarbonate carbon does lead to the incorporation of isotopic carbon in the citrate, but only in the tertiary carboxyl and not in other positions.

There are two known  $CO_2$  assimilation reactions leading to incorporation of  $CO_2$  into citrate carbon. These are the Wood and Werkman reaction (4) involving the reversal of oxalacetate decarboxylation, and the recently discovered reaction of Ochoa (5) involving the reversal of oxalosuccinate decarboxylation.



We may conclude, therefore, that the appearance of C<sup>13</sup> in the tertiary citrate carboxyl, either with isotopic acetoacetate as substrate or with non-isotopic acetoacetate and isotopic bicarbonate as substrates, is the

result of assimilation of isotopic  $\text{CO}_2$  via the Ochoa reaction (reversal of oxalosuccinate decarboxylation).

#### EXPERIMENTAL

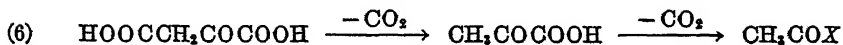
The isotopic acetoacetate was made either by the procedure described previously (1) or by a micro scale modification of the usual method, with metallic sodium as a condensing agent. The purity of the ester so obtained as determined from the refractive index and assay by the Van Slyke (6) method, ranged in several preparations between 85 and 95 per cent. The isotopic sodium bicarbonate was prepared by absorption of isotopic  $\text{CO}_2$  in 1 M  $\text{CO}_2$ -free NaOH, followed by precipitation with alcohol.

The kidney and brain tissues, rapidly excised from rats fasted 24 hours, were homogenized by the Potter-Elvehjem procedure; the skeletal muscle was prepared in the Latapie mincer. When not otherwise indicated in the text, the incubation of substrates and the isolation of products were carried out as described in the previous report (1). Each of the experiments of Tables I and II actually represents the combined values of two to six separate experiments carried out on successive days. This procedure was necessary to obtain sufficient citrate, of which a minimum of about 50 mg. was necessary for the isolation and degradation procedures.

#### DISCUSSION

Although the data of these experiments provide definite evidence for the conversion of acetoacetate to citrate in a variety of animal tissues, they also reveal that a substantial and, in brain for example, a major portion of the citrate arises without participation of the isotopic acetoacetate. That such citrate is formed via endogenous acetoacetate is excluded because of the non-dilution of the added isotopic acetoacetate.

Although the source of this non-isotopic citrate is still uncertain, there is apparently no reason to assume that it has been formed by a mechanism other than the one postulated for the formation of the isotopic citrate; namely, a  $\text{C}_2$ - $\text{C}_4$  condensation of oxalacetate with an acetyl group. The non-isotopic acetyl groups required could have been derived either from the oxidation of endogenous substrates, or from oxalacetate itself via pyruvate, according to equation (6).



#### SUMMARY

When acetoacetate, tagged with  $\text{C}^{13}$  in the  $\beta$  and carboxyl positions, is metabolized anaerobically in kidney and aerobically in brain and muscle, the citrate which accumulates has a high  $\text{C}^{13}$  excess in the primary carboxyl

positions and a low  $C^{13}$  excess in the tertiary carboxyl. The aerobic metabolism of non-isotopic acetoacetate in the presence of isotopic bicarbonate leads to assimilation of  $CO_2$  exclusively in the tertiary citrate carboxyl.

The variable  $C^{13}$  excess appearing in the primary carboxyls indicates that the citrate is formed partly from acetoacetate carbon and partly from endogenous substrates or oxalacetate. It is suggested that both types of citrate result from the same reaction; *i.e.*, the isotopic citrate by condensation of oxalacetate with isotopic acetyl groups from the added acetoacetate and the non-isotopic citrate by condensation of oxalacetate with non-isotopic endogenously formed acetyl groups.

The presence of  $C^{13}$  in the tertiary citrate carboxyl is assumed to result from  $CO_2$  assimilation via  $\alpha$ -ketoglutarate carboxylation (Ochoa reaction).

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# THE SYNTHESIS OF 1-METHYL-3-CARBOXYLAMIDE-6-PYRIDONE FROM TRIGONELLINE

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(Received for publication, September 13, 1947)

The isolation of 1-methyl-3-carboxylamide-6-pyridone from human urine as a metabolite of nicotinamide recently has been described by Knox and Grossman (1). These workers prepared the pyridone by the enzymatic oxidation of N<sup>1</sup>-methylnicotinamide with a quinine-oxidizing enzyme obtained from rabbit liver (1).

In connection with studies in these laboratories on the fate of N<sup>1</sup>-methylnicotinamide in man and in laboratory animals, 1-methyl-3-carboxy-6-pyridone was prepared by an alkaline ferricyanide oxidation of either trigonelline or N<sup>1</sup>-methylnicotinamide and converted to the corresponding amide, 1-methyl-3-carboxylamide-6-pyridone, by treatment with SOCl<sub>2</sub> and NH<sub>3</sub>. The experimental details of the synthesis are described here.

1-Methyl-3-carboxy-6-pyridone previously has been prepared from coumalic acid by ring closure with methylamine (2).

*Preparation of 1-Methyl-3-carboxy-6-pyridone*—To 8 gm. of trigonelline acid sulfate dissolved in 115 ml. of 2.5 N NaOH, there were added, at room temperature with stirring, 87 ml. of a 32 per cent solution of K<sub>3</sub>Fe(CN)<sub>6</sub> at the rate of 1 ml. per minute. After being stirred for an additional 45 minutes, the solution was adjusted to pH 3.5 with H<sub>2</sub>SO<sub>4</sub>, cooled to 50°, and the bluish crystalline mass filtered off and washed with cold water. An additional quantity of the material may be obtained by extraction of the filtrate with ether in a continuous extractor. The crystals were dissolved in 85 ml. of boiling water, stirred for a few minutes with 130 mg. of norit A, and filtered through a steam-heated funnel. The clear filtrate, on cooling overnight in the refrigerator, yielded 4.4 gm. of white needles melting at 240–241°.

*Preparation of 1-Methyl-3-carboxylamide-6-pyridone*—4 gm. of 1-methyl-3-carboxy-6-pyridone were heated under reflux for 1 hour on a steam bath with 20 ml. of SOCl<sub>2</sub>. After removal of the excess SOCl<sub>2</sub> by vacuum distillation, the solid acid chloride was shaken for a few minutes with 10 ml. of ice-cold concentrated NH<sub>4</sub>OH and the mixture placed in the refrigerator for about 1 hour. After filtering and washing with ice water there were obtained 3.0 gm. of the white crystalline compound. The melting point



after recrystallization from 5 ml. of water was 210–210.5° (in a bath previously heated to 190°). The mixed melting point with the pyridone amide isolated from human urine was unchanged.

#### SUMMARY

A procedure is described for the synthesis of 1-methyl-3-carboxylamide-6-pyridone, a metabolite of nicotinamide. Trigonelline or N<sup>1</sup>-methyl-nicotinamide was oxidized to 1-methyl-3-carboxy-6-pyridone by treatment with alkaline ferriocyanide. The acid pyridone was converted to the corresponding amide with SOCl<sub>2</sub> and NH<sub>3</sub>.

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# POLAROGRAPHIC DETERMINATION OF CYTOCHROME *c*\*

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and the Department of Anatomy, Washington University  
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(Received for publication, July 19, 1947)

In our studies on epidermal carcinogenesis in mice the small amounts of tissue available for analysis (1, 2) prompted a study of a new procedure for the quantitative determination of cytochrome *c*. For this purpose, we developed a polarographic procedure, based upon the fact that this enzyme gives a catalytic wave in a solution of  $\text{NH}_4\text{Cl}$ ,  $\text{NH}_4\text{OH}$ , and hexammino cobaltic chloride. The polarographic effect of proteins which yield catalytic waves was discovered by Heyrovský and Babicka (3), and extensively studied by Brdička (4-7). Müller and Davis (8) have recently proposed the protein index as a convenient method of characterizing and comparing the catalytic waves obtained with blood proteins and their degradation products.

## Method

*Catalytic Wave of Cytochrome c*—For a study of the catalytic wave an electrophoretically prepared sample of cytochrome *c* (Fe content 0.411 per cent) was employed. This was kindly given to us by Dr. H. Theorell of the Biochemical Institution of the Medical Nobel Institute, Stockholm, Sweden. Polarography of the cytochrome *c* was studied in a solution of 1 *N*  $\text{NH}_4\text{OH}$ , 1 *N*  $\text{NH}_4\text{Cl}$ , and 1 to  $1.1 \times 10^{-3}$  *M* hexammino cobaltic chloride containing 1 drop of a 1 per cent gelatin solution as a maximum suppressor per 10 ml. of solution. The mixture in an open shell vial, which permitted deep insertion of the mercury electrode, was polarographed immediately after mixing the cytochrome *c* with the other reagents, starting at  $-1.0$  volt (*versus* the saturated calomel electrode). The type of catalytic wave given by cytochrome *c* at a concentration of 18  $\gamma$  per ml. is shown in 1, Fig. 1. The tip of the catalytic wave is at about  $-1.5$  volts *versus* the saturated calomel electrode. The catalytic wave immediately follows the reduction of the cobaltous ion, and

\* This investigation was aided by grants from The International Cancer Research Foundation, the National Cancer Institute, and the Charles F. Kettering Foundation. Presented, in part, before the Thirty-eighth annual meeting of the American Society of Biological Chemists at Chicago, May, 1947.

after rising steeply to a maximum, drops rapidly, terminating only slightly above that of the inflection due to the reduction of the cobalt ion.

The height of the catalytic wave was measured from the tip of the wave to the point of inflection of the reduction of the cobalt ion. This was done by drawing a line contiguous with and parallel to that of the rise of the cobalt wave, and a line was drawn in a similar fashion through the first portion of the catalytic wave. At the point of intersection of these two lines a line was drawn parallel to the tip of the catalytic wave, and the distance in mm. was measured with a caliper. One could also determine the height of the cobalt wave alone and subtract this from the total height as measured from the base-line (residual current) to the tip of the

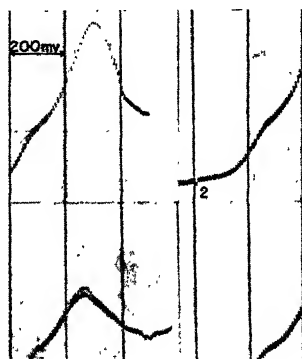


FIG. 1. The first vertical line on each section of Figs. 1 and 3 to 5 corresponds to  $-1.0$  volt versus the saturated calomel electrode. Polarograms of cytochrome *c* ( $18 \gamma$  per ml.), 1; of partially purified cytochrome *c* from rat kidney, 2; purified cytochrome *c* from rat kidney, 3; and catalase ( $80 \gamma$  per ml.), 4.

wave. Because the level of the base-line varied considerably from sample to sample, thus introducing greater errors, the latter procedure was not employed.

For a calibration curve the height of the catalytic wave in mm. was plotted against the concentration of cytochrome *c* in micrograms per ml. (Fig. 2). The wave height is proportional to concentration up to about  $30 \gamma$  per ml. and approaches a plateau at  $50$  to  $60 \gamma$  per ml. For these and subsequent data a Heyrovský polarograph, model XI (E. H. Sargent and Company) was employed. A galvanometer sensitivity of one-fiftieth (current sensitivity setting at 50) of the full sensitivity was used.

*Isolation of Cytochrome c from Tissues*—For the polarographic determination of cytochrome *c* the enzyme must be in a rather pure state

because other proteins mask the catalytic wave. For the initial extraction of the pigment the procedure of Rosenthal and Drabkin (9) was the most suitable, even though sufficient protein other than cytochrome *c* remained in the extract to prevent formation of a perfect catalytic wave. The following method has been found ideal for the isolation of the enzyme for polarography: The cytochrome *c* extract from tissues (Rosenthal and Drabkin (9)) is brought to pH 7.2 to 7.4 with dilute NaOH, centrifuged, and then adsorbed on aluminum oxide (Baker and Adamson's No. 1 or

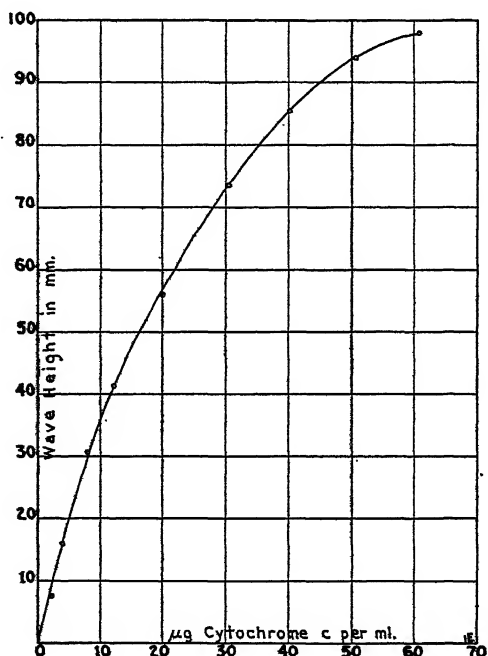


FIG. 2. Calibration curve of the catalytic wave of cytochrome *c* in  $N$   $NH_4Cl$ ,  $N$   $NH_4OH$ , and  $1 \times 10^{-3}$   $M$  hexammino cobaltic chloride.

1D or Baker's No. 12728). The adsorption is carried out in a 15 ml. centrifuge tube by thoroughly mixing the adsorbent and solution with a stirring rod. The cytochrome is adsorbed immediately, while most of the proteins remain in solution. The tube is then centrifuged, the supernatant decanted, and the adsorbent washed with 4 ml. of phosphate buffer (0.1  $M$   $NaH_2PO_4$ , 0.1  $M$   $Na_2HPO_4$  of pH 6.8 to 7.0) by stirring vigorously with the same stirring rod as in the first step and then centrifuging. This step is performed four more times in order to wash out the non-adsorbed proteins. The cytochrome *c* is then eluted with four successive

2 ml. portions of 2 *N*  $\text{NH}_4\text{OH}$  by agitating the alumina in the alkaline solution with the stirring rod and centrifuging and decanting the eluted cytochrome *c*. The supernatant containing the cytochrome *c* is chilled in an ice bath and then brought to pH 5.8 to 6.0 with 6 *N*  $\text{HCl}$  (and 0.5 *N*  $\text{HCl}$  or 0.2 *N*  $\text{NaOH}$  near the end-point) at which some material (largely aluminum hydroxide) flocculates. The mixture is heated at  $56^\circ$  for 15 minutes, and the material is then separated from the cytochrome *c* by centrifuging. The cytochrome *c* thus obtained gives perfect catalytic waves for mouse heart, kidney, and skeletal muscle and for rat heart and skeletal muscle. However, rat and mouse liver and rat kidney still contain some material which interferes with the catalytic wave. In 2, Fig. 1, is shown a polarogram of cytochrome *c* after the first adsorption and elution from a rat kidney. The wave is not perfect, since the tail of the wave does not drop to the point of inflection of the reduction of the cobalt ion. In tissue extracts not treated with aluminum oxide the catalytic hump is very small and the cobalt wave coalesces with that of the ascending portion of the wave owing to reduction of some interfering substance; *i.e.*, the wave due to the reduction of the cobalt ion is completely masked. The interference is not due to the formation of a catalytic wave, but rather to reduction which occurs at about  $-1.2$  to  $-1.3$  volts *versus* the saturated calomel electrode, and raises considerably the catalytic hump itself (2, Fig. 1).

The substance which interferes in mouse and rat liver and in rat kidney can be removed by a second adsorption on alumina as follows: The solution containing the cytochrome *c* after the first elution is brought to pH 5.8 to 6.0, centrifuged, and decanted into a graduated 15 ml. centrifuge tube. The enzyme is then precipitated with trichloroacetic acid and  $(\text{NH}_4)_2\text{SO}_4$ , if necessary (Rosenthal and Drabkin (9)), and the solution placed in the ice box overnight or for several days. The cytochrome *c* is spun down, dissolved in a few ml. of water, brought to pH 7.2 to 7.4, and then adsorbed on aluminum oxide. The tube is centrifuged, the supernatant decanted, and the alumina washed twice with phosphate buffer as on the first adsorption. The cytochrome *c* is then eluted as before, neutralized to pH 6.8 to 7.0 with 6 *N*  $\text{HCl}$  as previously, and the material including aluminum hydroxide which flocculates is centrifuged (after heating at  $56^\circ$  for 15 minutes). The supernatant is delivered into a 10 ml. volumetric flask, made to volume, and an aliquot taken for polarography. 3, Fig. 1 shows a polarogram of such an extract from rat kidney. The catalytic wave is perfect for this concentration of cytochrome *c*. (The ascending and descending portions of the catalytic wave are closer together as the concentration is increased.) 3, Fig. 1 corresponds to about 8  $\gamma$  per ml., while that of 1, Fig. 1 corresponds to 18  $\gamma$  per

ml. It is therefore quite apparent that the polarograph not only suffices for the quantitative determination of cytochrome *c*, but also enables one to follow its purification from an examination of the wave. Whether the interfering material in mouse and rat liver and in rat kidney is directly associated with cytochrome *c* or is very similar to it with respect to adsorptive properties on alumina remains to be seen.

The eluates obtained from either the first or second adsorptions have appreciable quantities of  $\text{NH}_4\text{Cl}$ , and the calibration curve must be made under similar conditions. The calibration curve (Fig. 2), in which the concentration is proportional to the wave height, was made with quantities of  $\text{NH}_4\text{Cl}$  which were used in the work reported in this paper, and the effect was negligible. Only at higher concentrations of cytochrome *c* does excess  $\text{NH}_4\text{Cl}$  affect the wave height and is therefore not important from an analytical standpoint, since only that portion of the curve is used in which the concentration is proportional to the wave height. The concentration of  $\text{NH}_4\text{Cl}$  at lower concentrations of cytochrome *c* must not be very great, since it causes confluence of the cobalt reduction wave with the ascending portion of the catalytic wave, thus making accurate measurement of the wave height more difficult.

*Specificity of Catalytic Wave*—To determine whether other amino acids might produce a catalytic wave in  $\text{NH}_4\text{Cl}$ ,  $\text{NH}_4\text{OH}$ , and hexammino cobaltic chloride, solutions of amino acids<sup>1</sup> (0.1 mg. per ml.), of aspartic acid, glycine, histidine, leucine, lysine, alanine, tryptophan, serine, valine, isoleucine, phenylalanine, proline, threonine, hydroxyproline, norleucine, tyrosine, arginine, glutamic acid, methionine, cysteine, cystine, and the tripeptide glutathione, were tested. Only cysteine, cystine, and glutathione gave catalytic waves, the former two reaching a maximum height at about  $-1.75$  volts and the latter at about  $-1.1$  volts *versus* the saturated calomel electrode. It is apparent then that the catalytic wave of cytochrome *c* and of the other proteins to be discussed later is due to  $-\text{SH}-$  or to  $-\text{S}-\text{S}-$  groups in the protein molecule (4, 10).

### Results

The results on the quantitative determination of cytochrome *c* in some mouse organs are shown in Table I. The average value in micrograms per gm. of fresh heart, kidney, liver, and skeletal muscle is respectively 543, 638, 227, and 84. In Table II the content in some rat tissue is given. For comparison the data obtained by Crandall and Drabkin (11), Potter and Dubois (12), and Stotz (13) are shown. In connection with the spectrophotometric determination of cytochrome *c* ideal conditions can

<sup>1</sup> The author is grateful to Mr. A. L. Caldwell of the Lilly Research Laboratories for samples of amino acids.

be obtained by carrying out the first adsorption and elution as described above. The solutions thus obtained are optically clear. Moreover by

TABLE I  
*Cytochrome c Content, in Micrograms per Gm. of Wet Weight of  
Tissues, in Mice*

Heart	Kidney	Liver	Skeletal muscle
482	565	202	113
846	594	201	103
586	738	301	110
773	839	301	88
435	885	228	77
362	666	237	91
487	508	247	77
415	519	214	76
502	577	217	46
	539	158	59
	542	204	
	750	255	
	573	220	
		191	
Average 543	638	227	84

TABLE II  
*Cytochrome c Content, in Micrograms per Gm. of Wet Weight, of Rat Tissues*

Heart	Kidney	Liver	Skeletal muscle
			87
300	326	160	124
442	297	190	118
355	320	164	125
336	269	100	113
350	296		77
			92
Average 356	302	154	105
" * 447	352	223	98
" † 371	247	90	97
" ‡ 530	330	68	160

\* From Crandall and Drabkin (11).

† From Potter and DuBois (12).

‡ From Stotz (13).

reprecipitation after the first adsorption the cytochrome *c* from a large amount of tissue can be concentrated to a very small volume.

That the polarographic method is adaptable to small amounts of tissue is illustrated in Table III. The tissues are extracted with small amounts of  $\text{NH}_4\text{OH}$ ,  $\text{H}_2\text{SO}_4$ , and water (Rosenthal and Drabkin (9)), and usually the addition of  $(\text{NH}_4)_2\text{SO}_4$  together with trichloroacetic acid is necessary to precipitate quantitatively the small amounts of cytochrome *c* present.

TABLE III  
*Cytochrome c Content of Tissues in Mice*

Wet weight of tissue per analysis	Heart	Kidney
gm.	$\gamma$ per gm.	$\gamma$ per gm.
0.075		547
0.116		603
0.074		527
0.118		393
0.139		439
0.134		410
0.142		380
0.109	431	
0.114	324	
0.090	322	
0.112	312	
Average.....	347	471

TABLE IV  
*Recovery of Added Cytochrome c*

Tissue	Cytochrome <i>c</i> in tissue extract	Cytochrome <i>c</i> added	Cytochrome <i>c</i>	
			Total	Recovered
	$\gamma$	$\gamma$	$\gamma$	$\gamma$
Heart.....	60	77	137	140
" .....	225	77	302	300
" .....	215	77	292	285
" .....	205	193	398	400
" .....	157	193	350	340
" .....	210	96	306	295
Kidney.....	470	96	566	560
" .....	470	160	630	630
" .....	480	160	640	630

The average values (Table III) are somewhat lower than those shown in Table I but are within the range of those in the latter table.

Good recoveries of pure cytochrome *c* were obtained when it was added to extracts of heart and kidney (as prepared previously) just prior to adsorption on alumina (Table IV). No attempt was made to test the re-



covery of cytochrome *c* prior to adsorption, since this had already been done by Rosenthal and Drabkin (9).

*Catalytic Waves of Other Highly Purified Proteins*<sup>2</sup>—Since the polarograph had proved to be of great value in the purification and determination of cytochrome *c*, other highly purified proteins were studied under exactly the same conditions as were used for cytochrome *c*. The following proteins were studied in the concentration (per ml.) indicated: catalase 80  $\gamma$ , ascorbic acid oxidase 20  $\gamma$ , tyrosinase 1200 Miller-Dawson catecholase units, D-glyceraldehyde-3-phosphate dehydrogenase 1.4 mg. of protein nitrogen, aldolase 1.5 mg. of protein nitrogen, prolactin 12  $\gamma$ , pepsin 8  $\gamma$ , pepsinogen 8  $\gamma$ , desoxyribonuclease 8  $\gamma$ , and carboxypeptidase 39  $\gamma$ . Pepsin, pepsinogen, catalase, aldolase, D-glyceraldehyde-3-phosphate

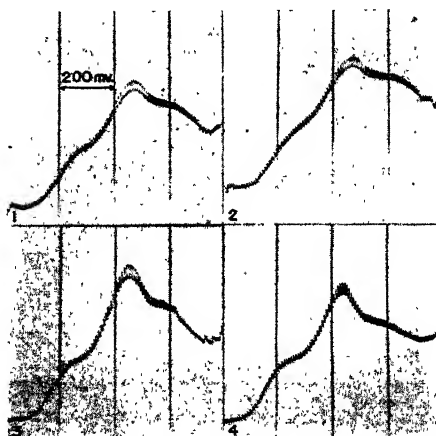


FIG. 3. Polarograms of ascorbic acid oxidase, 1; tyrosinase, 2; D-glyceraldehyde-3-phosphate dehydrogenase, 3; and aldolase, 4.

dehydrogenase, and carboxypeptidase were crystalline. The catalytic wave of catalase (4, Fig. 1) is quite similar to that of cytochrome *c*, but catalase gives a wave height of only one-tenth that of an equal weight of cytochrome *c*.

<sup>2</sup> The author is indebted to the following for the highly purified proteins: Dr. A. L. Dounce, School of Medicine and Dentistry, The University of Rochester, Rochester, New York, for the catalase, Dr. J. M. Nelson, Department of Chemistry, Columbia University, for ascorbic acid oxidase and tyrosinase, Dr. S. Velick, Washington University School of Medicine, for D-glyceraldehyde-3-phosphate dehydrogenase and aldolase, Dr. A. White, Yale University School of Medicine, for prolactin, Dr. J. H. Northrop, The Rockefeller Institute for Medical Research, for pepsinogen, Dr. J. D. Porsche, Armour and Company, for pepsin, Dr. M. McCarty, The Rockefeller Institute for Medical Research for desoxyribonuclease, and Dr. M. L. Anson of Continental Foods for carboxypeptidase.

In Fig. 3, 1 and 2 show respectively the catalytic waves of ascorbic acid oxidase and tyrosinase. These waves are somewhat similar. 3 and 4, Fig. 3, show the catalytic waves of the D-glyceraldehyde-3-phosphate dehydrogenase and aldolase respectively. These waves are again similar, but distinct from those of ascorbic acid oxidase and tyrosinase and also from that of cytochrome *c*.

In Fig. 4 the polarograms of prolactin, pepsin, pepsinogen, and desoxyribonuclease are shown in 1, 2, 3, and 4 respectively. In general these waves are similar to each other but signally different from all the preceding ones. Carboxypeptidase (Fig. 5) is different from all other proteins examined in that the tail of the wave does not drop. The catalytic waves of the last five proteins are essentially double in nature, a small

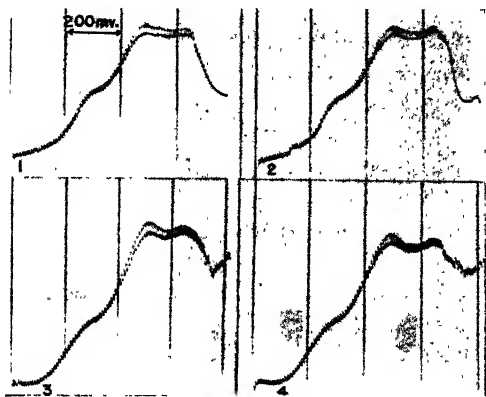


FIG. 4.

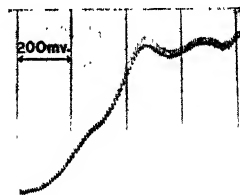


FIG. 5.

FIG. 4. Polarograms of prolactin, 1; pepsin, 2; pepsinogen, 3; and desoxyribonuclease, 4.

FIG. 5. Polarogram of carboxypeptidase.

catalytic hump occurring at  $-1.1$  volts and another at  $-1.7$  volts *versus* the saturated calomel electrode.

Although these catalytic waves are highly specific for  $-S-H-$  or  $-S-S-$  groups, it is quite apparent that there must be different arrangements or combinations of these groups in the proteins examined to result in such varied catalytic wave types.

Since the pH of the  $NH_4Cl$ ,  $NH_4OH$ , and hexammino cobaltic chloride solution is 9.35 (glass electrode), some of the proteins examined are probably denatured in spite of the fact that the solutions were polarographed within 10 minutes after the addition of the enzyme to the buffer solution. Therefore, it is possible that the catalytic waves may be due in part to denatured or partially denatured proteins.

For those proteins (enzymes) which yield catalytic waves it should be possible to work out extraction procedures whereby their quantitative determination on an absolute basis can be accomplished. As with cytochrome *c*, the technique may also be of value in a study of the purification of a protein from a study of the catalytic wave in the process of isolation.

#### SUMMARY

1. A polarographic method has been developed for the determination of cytochrome *c* in small amounts of tissue. The procedure is based upon the fact that cytochrome *c* gives a catalytic wave in a solution of ammonium chloride, ammonium hydroxide, and hexammino cobaltic chloride. The height of the catalytic wave is proportional to the concentration of cytochrome *c* in micrograms (1 to 30) per ml.

2. A new procedure for the purification and isolation of cytochrome *c* by adsorption on and elution from aluminum oxide is given.

3. A study of the catalytic wave is also of use in following the purification of cytochrome *c* at different steps in its isolation. Furthermore, work with the polarograph has shown that there are materials associated with cytochrome *c* in rat liver and kidney and mouse liver which have similar adsorptive properties to cytochrome *c*. These are lacking in heart and skeletal muscle of the rat and mouse and in mouse kidney.

4. Results obtained with rat and mouse kidney, liver, heart, and skeletal muscle are given and they are found to be in agreement with the spectrophotometric data reported by others.

5. The catalytic waves of some other highly purified proteins are given, and the possibility of using the polarograph for their quantitative determination on an absolute basis is briefly discussed. The instrument may also be of use as an aid in the purification of proteins (enzymes) from an examination of the catalytic wave obtained from tissue extracts under various conditions.

6. Only cysteine, cystine, and glutathione of the amino acids tested under the same conditions as for cytochrome *c* gave catalytic waves. Therefore the catalytic waves of the proteins examined must be due to —S—H— or —S—S— groups.

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# THE RÔLE OF POTASSIUM AND AMMONIUM IONS IN ALCOHOLIC FERMENTATION

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In the course of some experiments on the enzymes present in brewers' yeast maceration juice, it was observed that a brief dialysis of such a juice against distilled water greatly diminished or abolished its ability to ferment glucose. The activity could be completely restored by adding a boiled extract of the undialyzed maceration juice. Full activity could not be restored by the addition of  $\text{PO}_4^{=}$ ,  $\text{Mg}^{++}$ ,  $\text{Mn}^{++}$ , DPN, ATP, adenylic acid, or cocarboxylase when these were added singly or together.<sup>1</sup> However, it could be restored by adding either  $\text{K}^+$  or  $\text{NH}_4^+$  in optimal concentrations. Studies on the fermentation of HDP-glucose mixtures in the presence and absence of  $\text{K}^+$  showed that HDP was fermented in the absence of  $\text{K}^+$  or  $\text{NH}_4^+$ , but the fermentation of glucose was either abolished or greatly retarded.

The stimulating effect of  $\text{K}^+$  on glucose fermentation by a cell-free yeast extract has been reported by Farmer and Jones (1). Smythe (2) found that  $\text{NH}_4^+$  shortened the induction period when glucose was fermented by an extract of bakers' yeast. A number of workers have shown that  $\text{K}^+$  is necessary for maximum utilization of glucose by living yeast cells (3-7). Hevesy and Nielson, employing radioactive  $\text{K}^+$ , showed that a rapid  $\text{K}^+$  exchange between cells and the medium took place in actively fermenting yeast (4). Pulver and Verzář demonstrated that yeast cells took up  $\text{K}^+$  from the medium during a short period fermentation (5). This was confirmed by Conway and O'Malley (6) as well as by Rothstein and Enns (7), who observed that the  $\text{K}^+$  absorption seemed to be a cation exchange;  $\text{H}^+$  diffused out of the cells and  $\text{K}^+$  diffused in. The exchange could be predicted on the basis of a Donnan equilibrium. Similar changes occur in bacterial fermentations; Leibowitz and Kupermintz found that *Escherichia coli* takes up  $\text{K}^+$  from the medium during the early phase of glucose utilization and releases it later on (8).

<sup>1</sup> The following abbreviations are used throughout: DPN, diphosphopyridine nucleotide; ATP, adenosine triphosphate; HDP, fructose-1,6-diphosphate; DPT, diphosphothiamine; PGA, phosphoglyceric acid; FMP, fructose monophosphate.

The fact that  $K^+$  or  $NH_4^+$  is necessary for alcoholic fermentation by a cell-free yeast extract suggests that the rôle of these ions in living yeast is not simply that of furnishing readily diffusible cations.

#### EXPERIMENTAL

##### *Materials—*

Hexose diphosphate. Barium fructose-1,6-diphosphate obtained from the Schwarz Laboratories, Inc., New York. The sodium salt was prepared by removing the barium with  $H_2SO_4$  and neutralizing the acid to pH 6 with NaOH.

Adenosine triphosphate. Prepared from rabbit muscle as described by LePage (9). The sodium salt was used in these experiments.

Adenylic acid. Samples of both yeast and muscle adenylic acid were employed.

Succinate buffer (0.1 M, pH 6) and accessory factors. 2 ml. of 0.5 M succinic acid, 1.82 ml. of 1 N NaOH, 4 mg. of  $MgCl_2 \cdot 6H_2O$ , 4 mg. of  $MnCl_2 \cdot 4H_2O$ , 8 mg. of DPN, and 0.025 ml. of acetaldehyde, made up to 10 ml.

Diphosphopyridine nucleotide. Schwarz Laboratories cozymase.

Fructose-6-phosphate.<sup>2</sup> Prepared by acid hydrolysis of HDP according to Neuberg *et al.* (10). On the basis of the organic phosphate analysis the compound was  $C_6H_{11}O_9PBa \cdot 3H_2O$ .

Phosphoglyceric acid. Crystalline barium salt,  $C_3H_5O_7PBa \cdot 2H_2O$ .

Diphosphothiamine. Merck's cocarboxylase.

Yeast maceration juice. Dried yeast was prepared from a brewers' bottom yeast<sup>3</sup> by the procedure outlined by Nilsson and Alm, Fraction B (11). For the preparation of Lebedev juice, 1 part of finely ground yeast was mixed with 3 parts of distilled water and the mixture incubated at 37° for 1 hour. The suspension was then centrifuged for 20 to 30 minutes at 3000 R.P.M. in a No. 1 International centrifuge. Dialyzed yeast juice was prepared by putting 3 to 4 ml. of Lebedev juice into a 6 inch piece of 23/32 Visking casing and dialyzing against running distilled water for 90 minutes unless otherwise stated. This process was carried out in a crushed ice bath.

Boiled yeast juice. Yeast maceration juice, contained in a centrifuge tube, was put into boiling water and stirred for 1 minute. The coagulated proteins were removed by centrifuging.

##### *Methods*

All of the fermentations were carried out in Warburg flasks of the conventional design at pH 6. The gas phase was air in most of the experiments.

<sup>2</sup> We wish to thank Dr. M. F. Utter for a sample of this substance.

<sup>3</sup> The yeast, as drawn from the vat, was obtained from the Keeley Brewing Company, Chicago, Illinois.

At pH 6 there is a slight retention of  $\text{CO}_2$ . However, it is so small that it can be neglected.

The earlier experiments were carried out at  $38^\circ$ , but it was found that some of the enzymes were inactivated at this temperature, since the addition of extra substrate at the 60 minute interval did not improve the rate of  $\text{CO}_2$  formation, whereas, when the same experiment was carried out at  $30^\circ$ , the addition of extra substrate had a pronounced effect. Most of the experiments were carried out at  $30^\circ$ .

Yeast maceration juice in a long, flattened piece of Visking casing was dialyzed against distilled water contained in a 1 liter cylinder. The membrane was agitated continuously by bubbling air through the water. The efficacy of the dialysis in removing readily diffusible ions is illustrated by the fact that the inorganic phosphate concentration of the juice was changed from an initial value of 0.12 M to 0.004 M in 30 minutes and to 0.0008 M in 60 minutes.

Glucose determinations were made by Somogyi's method (12). The  $\text{ZnSO}_4$  used in this method was added to an aliquot of the fermentation mixture taken directly from the Warburg flask and served the dual purpose of stopping the reaction and furnishing the first step in the deproteinization. The method proved to be highly specific for glucose in these mixtures. Fructose-6-phosphate and HDP were quantitatively removed by the deproteinization procedure and presumably other phosphate esters are likewise removed.

Hexose monophosphates and HDP were separated by a modification of the scheme proposed by LePage and Umbreit (13): With the small amounts of esters that were formed in these experiments it was necessary to alter the procedure as follows: To 2 ml. of fermentation sample was added 0.2 ml. of 50 per cent trichloroacetic acid. After 30 minutes the mixture was centrifuged at 12,000 R.P.M. in lusteroid tubes in a high speed centrifuge. The supernatant fluid was quantitatively transferred to a graduated centrifuge tube and adjusted to the full red phenolphthalein end-point with approximately 0.5 N  $\text{Ba}(\text{OH})_2$ . Methyl alcohol was then added to the mixture, 1 volume for every 4 volumes of fluid, stirred thoroughly, and chilled in an ice water bath for 20 minutes. By this procedure the total volume was kept small and HDP was quantitatively precipitated. It was removed by centrifuging sharply for 10 minutes. The supernatant fluid, which contains the hexose monophosphates, was quantitatively removed and the volume made up to 10 ml. Fructose determinations were carried out on 2 ml. aliquots of this solution (14). The precipitate, which contains the HDP, was stirred with 2 ml. of 0.1 N  $\text{HCl}$  and the final volume made up to 10 ml. Fructose determinations were again made on 2 ml. aliquots. The final color production is sensitive to small changes in  $\text{HCl}$  concentration; therefore it is necessary to standardize the procedure carefully. Color in-



tensities were compared in a Coleman universal spectrophotometer at 490  $m\mu$ . A mixture of 5 micromoles of fructose-6-phosphate and 5.5 micromoles of HDP in 2 ml. of water was analyzed by this procedure. There were recovered 5.5 micromoles of fructose-6-phosphate and 5.4 micromoles of HDP. Without the methyl alcohol addition, only 25 to 50 per cent of the HDP was recoverable at these concentration levels.

Although the esters were quantitatively recoverable from simple solutions, fructose-6-phosphate behaved anomalously in the succinate buffer mixture used in our experiments. When it was added to this mixture, a part of the ester became precipitable by  $Ba^{++}$  and appeared in the HDP fraction. The amount that was precipitated constituted a large part of the ester when only small amounts were present. It was necessary to construct a curve relating the amount of fructose-6-phosphate added to the buffer mixture to the  $E$  values read on the Coleman spectrophotometer.

### *Results*

Yeast maceration juice, prepared from this type of dried brewers' yeast, did not ferment glucose unless a small amount of HDP was also added. Nor did it ferment HDP by itself, to any appreciable extent. Apparently the process of making the dry yeast destroys adenylypyrophosphatase and the juice does not contain adequate amounts of substances that can act as phosphate acceptors (15).

When both glucose and HDP are added, the fermentation proceeds according to the well known equation of Harden and Young, which states that 2 moles of  $CO_2$  and 1 mole of HDP are formed for every 2 moles of glucose fermented. The rate of fermentation as a function of time is shown in Fig. 1. At the end of 3 hours, the total  $CO_2$  evolved is close to the theoretical amount that can be obtained from 40 micromoles of glucose and 5 micromoles of HDP (896 c.mm.).

A brief dialysis against distilled water greatly reduced the ability of the juice to ferment the same glucose-HDP mixture, although inorganic phosphate was returned to the dialyzed juice as a mixture of the sodium phosphates,  $NaH_2PO_4$  and  $Na_2HPO_4$ . This reduction in activity occurred in spite of the fact that the well known cofactors,  $Mg^{++}$ ,  $Mn^{++}$ , and DPN, necessary for alcoholic fermentation, were returned to the dialyzed juice. The full activity was essentially completely restored by adding a boiled extract of the original, undialyzed juice or simply by adding some  $K^+$ , either as the phosphate or as the chloride. Abolition of the induction period when  $K^+$  or boiled juice was added, which is apparent in the curves shown in Fig. 1, was not a constant feature in the many similar experiments that were made.

In the early experiments, before it was found that  $K^+$  could restore the

activity, certain other factors such as adenylic acid, ATP, and DPT, alone or in combination, were added to the dialyzed juice so that the final concentration of each of these substances was 0.002 M. They had no stimulatory effect. On the basis of such experiments it was concluded that the enzymes present in the dialyzed juice had associated with them adequate amounts of these factors.

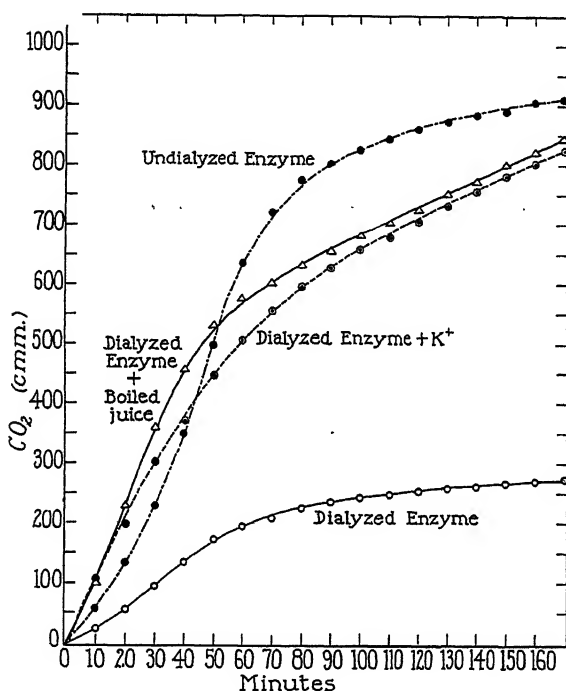


FIG. 1. Fermentation of glucose (40 micromoles) plus HDP (5 micromoles) by undialyzed and dialyzed maceration juice. Sodium phosphate (0.02 M, pH 6) was present in all vessels except those which received boiled yeast juice. Succinate buffer (0.4 ml.) was added to each vessel. Final concentrations of supplements, boiled juice, 0.2 ml. per vessel; K<sup>+</sup>, 0.02 M added as the phosphate. Of the dialyzed enzyme, 0.3 ml. was added per vessel, while with the undialyzed juice, 0.2 ml. was added. Final volume; 1 ml.; gas phase, air; *t*, 30°.

On the other hand, there was a slight improvement in the rate of fermentation when extra DPN was added. Magnesium (added as MgCl<sub>2</sub>) also stimulated the fermentation somewhat and manganese (added as MnCl<sub>2</sub>) was even better in this regard. Because of these facts, these substances were routinely added to the succinate buffer.

Fig. 1 shows that there was some fermentation of a glucose-HDP mixture

by dialyzed maceration juice in the absence of  $K^+$ . Further experiments showed that the amount of  $CO_2$  produced was roughly accounted for by the amount of HDP added, on the basis that each mole of HDP yields 2 moles of  $CO_2$ . Table I shows the over-all  $CO_2$  production when varying amounts of HDP were added, together with a constant amount of glucose, to dialyzed juice supplemented with either sodium phosphate, potassium phosphate, or boiled yeast juice. In the absence of  $K^+$  or boiled juice, the  $CO_2$  apparently is derived from HDP, while glucose acts as a phosphate acceptor and is converted to an ester which cannot be converted to HDP, at least not at an appreciable rate. On the other hand, when  $K^+$  or boiled juice is added to the system, glucose also is fermented to  $CO_2$ , as is evidenced by the large

TABLE I  
*Fermentation of Glucose by Dialyzed Yeast Maceration Juice*

Each vessel contained 0.1 M succinate, pH 6; 0.002 M  $MgCl_2$  and  $MnCl_2$ ; 0.005 M acetaldehyde, and 0.4 mg. of approximately 80 per cent DPN; final volume, 1 ml.

System			Supplement	Reaction time	$CO_2$ produced	Glucose utilized
Dialyzed Lebedev juice*	HDP	Glucose				
ml.	micromoles	micromoles		min.	c.mm.	micromoles
0.5	4	40	0.01 M Na phosphate, pH 6	120	169	8.6
0.5	4	40	0.25 ml. boiled yeast juice	120	860	32.4
0.3	5	40	0.01 M Na phosphate, pH 6	180	277	14.4
0.3	10	40	0.01 " " " " 6	180	493	24
0.3	5	40	0.01 " K " " 6	180	846	34
0.3	5	40	0.2 ml. boiled yeast juice	180	867	33.2

\* Lebedev juice prepared by incubating 5 gm. of dry powdered brewers' bottom yeast with 15 ml. of water at  $30^\circ$  for 50 minutes, centrifuging, and dialyzing the clear juice against ice-cold, running distilled water for 75 minutes.

increase in  $CO_2$  production and glucose utilization. These results suggest that the degradation of HDP to  $CO_2$  and ethyl alcohol goes on in the absence of  $K^+$  and this indicates that the phosphate transfers, (a) from 1,3-diphosphoglyceric acid to form ATP and (b) from phosphopyruvic acid to form ATP, proceeded normally.

Moreover, the experiments indicate that the inability of the enzyme system to ferment glucose results from a failure to transform glucose into HDP at an appreciable rate. If this is so, the monophosphoric acid esters of glucose should accumulate and the equilibrium ester, 75 per cent glucose-6-phosphate, 25 per cent fructose-6-phosphate, should be formed (16). We were unable to demonstrate that the mixed esters in these proportions were formed in our experiments. Varying amounts of fructose-6-phosphate were

incubated for 5, 10, and 20 minutes, and it was found that approximately 50 per cent had disappeared, whether the analysis was made at the 5 or at the 20 minute interval. Negligible amounts of the ester were fermented in this short period.

Several experiments were performed in which different mixtures of glucose and HDP were fermented in sodium phosphate buffer. The over-all production of  $\text{CO}_2$ , the glucose utilized, and the fructose phosphate formed were measured and are recorded in Table II. Although somewhat more  $\text{CO}_2$  is produced than can be accounted for as being derived from HDP, it

TABLE II

*Fermentation of Hexose Diphosphate in Sodium Phosphate Buffer*

Each vessel contained 0.4 ml. of succinate buffer, 0.1 M, pH 6; 0.1 ml. of sodium phosphate buffer, 0.2 M, pH 6; dialyzed Lebedev juice; 0.002 M  $\text{MgCl}_2$  and  $\text{MnCl}_2$ , and 0.4 mg. of approximately 80 per cent DPN; final volume, 1 ml.

Experiments 1 and 2, 0.3 ml. of dialyzed Lebedev juice per vessel; Experiment 3, 0.2 ml. of dialyzed Lebedev juice per vessel.

Experiment No.	Substrate		$\text{CO}_2$ formed	Glucose utilized	Barium soluble fraction (as FMP)	Hexose monophosphate*
	HDP	Glucose				
	<i>micromoles</i>	<i>micromoles</i>	<i>c.mm.</i>	<i>micromoles</i>	<i>micromoles</i>	<i>micromoles</i>
1. 240 min.	2.5	37	136	8.4	9.5	19
	5	37	327	20.4	12.4	24.8
	10	37	714	35.3	14.4	28.8
2. 240 "	2.5	37	118	8.5	10.3	20.6
3. 340 "	2	29	112	7.6	7.3	14.6
	4	29	216	15.3	10.3	20.6
	6	29	320	22.1	11.6	23.2
	8†	39	333	25.9	12.7	25.4
	10†	48	324	27.3	13.1	26.2

\* Based on the assumption that 50 per cent of the fructose monophosphate is converted to hexose monophosphate.

† Fermentation was still proceeding actively at the end of the experimental period.

is very much less than would be expected from the glucose utilization if an appreciable amount of glucose were fermented. Fructose monophosphate accumulated in all of these experiments; however, there is not good agreement between the amount of this ester and the glucose utilized. This is partly due to the fact that all of the chromogenic substance present in the barium soluble fraction was not fructose monophosphate, but also included some free fructose. In an experiment with fructose-6-phosphate which was separated from the mixture by the alcohol precipitation procedure of LePage and Umbreit (13), it was found that with increasing incubation time a progressively larger amount of the color-producing substance re-

mained soluble in alcohol. This would occur if a weak phosphatase action split the ester to free fructose.

The mixtures in the vessels which received 8 and 10 micromoles of HDP in Experiment 3 were still actively fermenting at the end of 340 minutes, and the low yields of  $\text{CO}_2$  suggest that the reactions had not gone to completion.

The results obtained in this experiment may be compared with those shown in Table III. In these experiments the fermentations of glucose-HDP mixtures were carried out in sodium phosphate buffer and in this same buffer supplemented with either potassium phosphate, ammonium phosphate, or boiled yeast juice. The marked stimulation of  $\text{CO}_2$  production

TABLE III  
*Fermentation of Glucose*

Each vessel contained 0.04 M succinate buffer, pH 6; 0.002 M  $\text{MgCl}_2$  and 0.002 M  $\text{MnCl}_2$  together with 0.4 mg. of approximately 80 per cent DPN. In Experiment 1, 0.6 ml. of dialyzed juice was used in a total volume of 2 ml., while in Experiment 2, 0.2 ml. of dialyzed juice was present in a total volume of 1 ml.

Sodium phosphate, 0.2 M, pH 6; potassium phosphate, 0.2 M in  $\text{K}^+$ , pH 6; ammonium phosphate, 0.2 M, adjusted to pH 6.

Experiment No.	Substrate		Supplement added	$\text{CO}_2$ formed	Glucose utilized	Barium soluble fraction (as FMP)	Barium insoluble fraction (as HDP)
	HDP	Glucose					
	micro-moles	micro-moles		c.mm.	micro-moles	micro-moles	micro-moles
1	3.2	38	0.2 ml. Na phosphate	103	9.3	8.5	1.7
	3.2	38	0.2 " K "	790	37.3	8.2	16.6
	3.2	38	0.4 " boiled yeast juice	943	34.7	6.8	17
2	3.3	28.6	0.1 " Na phosphate	129	12.2	9.2	2.3
	3.3	28.6	0.1 " K "	573	28.4	8.7	6
	3.3	28.6	0.025 ml. $\text{NH}_4$ phosphate	559	26.8	10	11

in those samples supplemented with  $\text{K}^+$ ,  $\text{NH}_4^+$ , or boiled juice is paralleled by a greater glucose utilization; nevertheless no more fructose monophosphate was found. Fructose analyses were also carried out on the barium insoluble fraction and it was found that this fraction was markedly increased in the supplemented samples. This is further proof that the addition of  $\text{K}^+$  or  $\text{NH}_4^+$  allows the fermentation of glucose to proceed normally to HDP, which begins to accumulate as the glucose is exhausted.

Further proof for the necessity of  $\text{K}^+$  or  $\text{NH}_4^+$  in the formation of HDP was acquired by showing that the 3-carbon intermediate phosphoglycerate was also fermented to  $\text{CO}_2$  with glucose present as the phosphate acceptor. As long as a dialyzed yeast juice and only sodium-containing buffers were

used, the  $\text{CO}_2$  seemed to be derived only from the phosphoglycerate. Fructose monophosphate accumulated in such mixtures. When the same mixtures were fermented in buffers supplemented with  $\text{K}^+$ , glucose was also fermented, as is evidenced by the enhanced  $\text{CO}_2$  production and the accumulation of a barium insoluble ester (HDP) (Table IV).

An experiment was also carried out with phosphopyruvate as the phosphate donor and glucose as the phosphate acceptor. Iodoacetate (0.004 M) was added to prevent the fermentation of glucose, and sodium fluoride (0.01 M) was also included in order to prevent the conversion of phosphopyruvate back to phosphoglycerate. Under these conditions it was found

TABLE IV  
*Fermentation of Sodium Phosphoglycerate*

Each vessel contained 0.04 M succinate buffer, pH 6, supplemented as in previous experiments. Dialyzed Lebedev juice (0.3 ml. per vessel) was used throughout; final volume, 1 ml.

Sodium phosphate, 0.2 M in  $\text{PO}_4^{3-}$ , pH 6; potassium phosphate, 0.2 M in  $\text{K}^+$ , pH 6.

Experiment No.	Substrate		Phosphate buffer added	$\text{CO}_2$ formed	Glucose utilized	Barium soluble fraction (as FMP)	Barium insoluble fraction (as HDP)
	PGA	Glucose					
	<i>micro-moles</i>	<i>micro-moles</i>		<i>c.mm.</i>	<i>micro-moles</i>	<i>micro-moles</i>	<i>micro-moles</i>
1	8.7	19.4	None	170			
	8.7	19.4	0.1 ml. Na phosphate	167	7.8	7.6	1
	8.7	19.4	0.1 " K "	565	19.2	3.0	6.4
2	5	18.4	0.1 " Na "	38	1.8	2.4	0.6
	5	18.4	0.1 " K "	485	18.4	3.2	2.8
3	10	18.4	0.1 " Na "	214	8.4	6.8	1.2
	15	18.4	0.1 " " "	342	14.2	13.0	1.6

that phosphopyruvate transferred its phosphate to glucose as readily in a sodium buffer as in one that contained  $\text{K}^+$  or  $\text{NH}_4^+$ .

In some of the experiments just described it was shown that ammonium ions were as efficacious as potassium ions in stimulating the fermentation of glucose. An experiment was designed to ascertain the concentration level of each of these ions that is necessary to give maximum activity. Table V shows that, whereas 0.01 M  $\text{K}^+$  is necessary for full activity, 0.005 M  $\text{NH}_4^+$  is equally effective. Other experiments showed that even 0.001 M  $\text{NH}_4^+$  restored the fermentation to 85 per cent of the maximum value over a 300 minute period. An interesting feature of the experiments with small amounts of  $\text{NH}_4^+$  was the observation that the induction period was considerably prolonged. With 0.001 M  $\text{NH}_4^+$  in the system, the fermentation of a given amount of substrate did not attain its maximum rate until 200

TABLE V

*Effect of Varying Amounts of  $K^+$  or  $NH_4^+$  on Fermentation of Glucose*

Vessels set up as before, 0.3 ml. of dialyzed Lebedev juice added to each one. In both experiments the phosphate concentration was 0.02 M;  $K^+$  was added as KCl,  $NH_4^+$  as ammonium phosphate; final volume, 1 ml.

Theoretical amount of  $CO_2$  obtainable in Experiment 1, 896 c.mm.; in Experiment 2, 447 c.mm.

Experiment No.	Substrate		Molarity of $K^+$ or $NH_4^+$	$CO_2$ produced c.mm.
	HDP micromoles	Glucose micromoles		
1. 280 min.	2.5	40	0.02 M $K^+$	827
	2.5	40	0.01 " "	782
	2.5	40	0.005 M $K^+$	415
	2.5	40	0.0025 M $K^+$	163
	2.5	40	Control in $Na^+$ buffer	118
2. 240 "	3.2	20	0.02 M $NH_4^+$	485
	3.2	20	0.01 " "	457
	3.2	20	0.005 M $NH_4^+$	455
	3.2	20	Control in $Na^+$ buffer	179

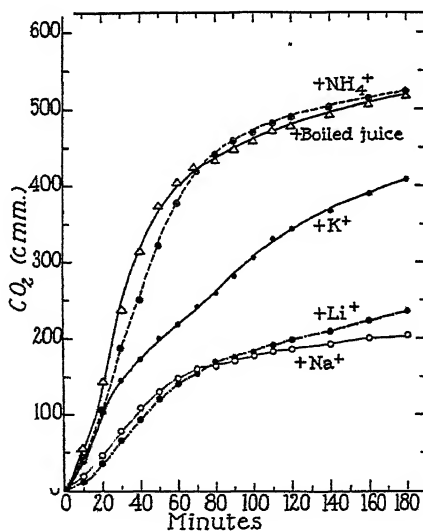


FIG. 2. Comparison of the fermentation of glucose (20 micromoles) plus HDP (3.7 micromoles) as influenced by various ions. Sodium phosphate (0.02 M, pH 6) was present in all vessels except those which received boiled yeast juice (0.2 ml.). Succinate buffer (0.4 ml.) was added to each vessel.  $NH_4^+$ ,  $K^+$ , and  $Li^+$  were added as the chlorides, and the final concentration of each ion was 0.005 M. Final volume, 1 ml.; gas phase, air;  $t$ , 30°.

minutes, but with 0.003 M  $\text{NH}_4^+$ , maximum  $\text{CO}_2$  production was observed at 120 minutes. Smythe (2) observed a similar phenomenon in his experiments.

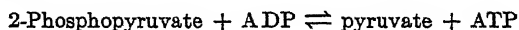
Lithium ions, like sodium ions, do not stimulate the fermentation of glucose. This is shown in Fig. 2. In this experiment a suboptimal amount of  $\text{K}^+$  (0.005 M) was used. Ammonium ions in the same concentration were as effective as boiled yeast juice.

It should be emphasized that  $\text{NH}_4^+$  or  $\text{K}^+$  does not act by protecting the enzymes from inactivation during these long experiments. Mixtures of glucose and HDP were fermented in buffers containing only  $\text{Na}^+$ . At the 60, 120, and 180 minute intervals,  $\text{K}^+$  (to give 0.01 M in the vessels) was tipped into the mixtures from the other side arm of the Warburg flasks. In each case fermentation of glucose ensued.

#### DISCUSSION

All of the results are consonant with the hypothesis that ammonium or potassium ions are necessary for the formation of hexose diphosphate from hexose monophosphate as catalyzed by a dialyzed yeast maceration juice prepared from brewers' bottom yeast. This probably is the reason for the frequently reported observation that either of these ions is required by living yeast cells in order to obtain maximum fermentation of glucose. The necessity for either  $\text{K}^+$  or  $\text{NH}_4^+$  in this cell-free juice makes the hypothesis of Conway and O'Malley (6) less attractive. These authors explained the beneficial effects of these ions on living yeast cells on the basis of a Donnan equilibrium. The yeast cell was assumed to be permeable to  $\text{H}^+$ ,  $\text{K}^+$ ,  $\text{NH}_4^+$ , and organic acid anions but impermeable to  $\text{Na}^+$ . In their view,  $\text{K}^+$  was utilized to maintain the ionic equilibrium within the cell as  $\text{H}^+$  diffused out. It now seems more likely that at least one of the functions of  $\text{K}^+$  or  $\text{NH}_4^+$  within the cell is to facilitate the phosphorylation of hexose monophosphate.

The yeast juice differs from the muscle extract studied by Boyer, Lardy, and Phillips (17, 18) in that these authors reported that  $\text{K}^+$  or  $\text{NH}_4^+$  was necessary for the transfer of phosphate from 2-phosphopyruvate to adenosine diphosphate.



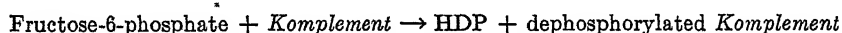
Lardy and Ziegler (19) pointed out that such muscle extracts when dialyzed for 78 hours were still able to carry out this phosphorylation without added  $\text{K}^+$  at about three-sevenths of the maximum rate observed when  $\text{K}^+$  was added.

Dialyzed yeast juice is able to ferment either HDP or PGA without added  $\text{K}^+$  or  $\text{NH}_4^+$ . In the process glucose is phosphorylated and therefore the transfer of phosphate from 2-phosphopyruvate proceeds normally in the



absence of these ions. Under these conditions hexose monophosphates accumulate, as is evidenced by an increase in substances giving the fructose color reaction. The lack of agreement between the amount of hexose monophosphates formed, as calculated from the fructose color value, and the glucose utilization, when only HDP is being fermented, may be explained on the basis of a slow phosphatase reaction. Over the long experimental period (240 minutes) some of the fructose-6-phosphate is hydrolyzed to free fructose. Fructose gives about twice as much color by Roe's method as does an equivalent amount of fructose-6-phosphate. Since all the hexose monophosphate values are calculated from a curve which was constructed by using fructose-6-phosphate, the determined values for these phosphate esters would be too high.

When optimal amounts of  $K^+$  or  $NH_4^+$  were added to the buffer, the fermentation of glucose proceeded at a rapid rate with little or no induction period. In several experiments it was observed that there was a prolonged period before the maximum fermentation rate was established when sub-optimal amounts of the ions were added. This suggests that these ions in some way facilitate the production of another substance (cofactor) which is perhaps necessary for the formation of HDP. Ohlmeyer (20) has reported that a new cofactor, *Komplement*, is necessary for the phosphohexokinase reaction.



He pointed out that ATP could perform this function, although it was not as efficient as *Komplement*. In the introduction to his paper he also stated that in a buffer containing ammonium phosphate fermentation of glucose was carried out by dialyzed yeast juice without adding either ATP or *Komplement*. In order to demonstrate the need for a new cofactor he dialyzed the yeast juice for long periods (24 hours). It is unlikely that our brief 90 minute dialysis would remove a cofactor completely, although it might reduce the concentration to a suboptimal level. This occurs with DPN for example, since a slight improvement in the rate of fermentation was observed when more of this factor was returned to the dialyzed juice.

Potassium does not appear to act by antagonizing sodium ions in our system. Large amounts of NaCl were added to mixtures which were actively fermenting glucose in the presence of 0.02 M  $K^+$ . The concentration of NaCl could be increased up to 0.08 M without impairing the over-all fermentation. Larger amounts (*i.e.* 0.2 M) had an inhibitory effect.

#### SUMMARY

1. A dialyzed yeast maceration juice prepared from brewers' bottom yeast has been shown to catalyze the fermentation of hexose diphosphate

and of phosphoglycerate in a sodium phosphate buffer to which no  $K^+$  or  $NH_4^+$  has been added, but which contains a suitable phosphate acceptor such as glucose.

2. In systems that contain no  $K^+$  or  $NH_4^+$ , the glucose is converted to hexose monophosphates, and these esters accumulate as the fermentation proceeds.

3. When either  $K^+$  or  $NH_4^+$  is added to the fermentation mixture, the glucose is also fermented and the yield of  $CO_2$  corresponds closely to that which should be obtained according to the equation of Harden and Young.

4. This suggests that either of these two ions is necessary for the formation of hexose diphosphate from hexose monophosphate as catalyzed by a dialyzed yeast maceration juice.

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# A STUDY OF THE ACTION OF PURIFIED AMYLASE FROM ASPERGILLUS ORYZAE, TAKA-AMYLASE\*

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The amylase produced by the fungus, *Aspergillus oryzae*, has been used in the Orient for centuries and as a partially purified product since 1898 (1), but surprisingly little is known of its action and properties. This lack of quantitative information is probably due largely to the fact that many investigations have dealt with rather crude alcohol precipitation products of extracts of the mold which contained relatively high concentrations of other carbohydrases as well as amylase.

The investigation reported here deals with a study of the products formed from starch by highly purified taka-amylase (2). The preparations of the amylase gave no evidence of the presence of maltase activity when the highest concentrations used here were allowed to react with 1 per cent maltose for 24 hours under the conditions used for the hydrolysis of starch (3).

## EXPERIMENTAL

Lintner's soluble potato starch was hydrolyzed at 40° under conditions (0.05 M sodium chloride, 0.01 M acetate, and pH 5.0) which had been found to favor the stability and activity of the amylase (3). In experiments designed to study the extent of the hydrolysis of starch, concentrations of amylase were chosen so that the reactions would proceed rapidly and be practically complete before contamination by yeasts and bacteria might be expected appreciably to influence the results.

Aliquots were removed at intervals from the reaction mixtures and measured for reducing values. These values were determined iodometrically (4) and converted to their equivalents of maltose. They are reported in terms of the percentage yield of the maltose which could be obtained theoretically from the starch.

## Results

*Extent of Hydrolysis of Starch*—The data given in Fig. 1 show that increasing concentrations of taka-amylase increase the initial velocity of the

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† This work is taken from a dissertation submitted by Gertrude D. Werner in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Chemistry under the Faculty of Pure Science, Columbia University.

hydrolysis of starch and also the extent of the hydrolysis obtained after prolonged action. The reaction curves are typical of enzyme action in general in that they exhibit two stages of the hydrolysis, a period of rapid increase in reducing values followed by one in which the reducing values rise only very slowly. However, in this work, the extent of the hydrolysis of starch depends within wide limits upon the concentration of amylase

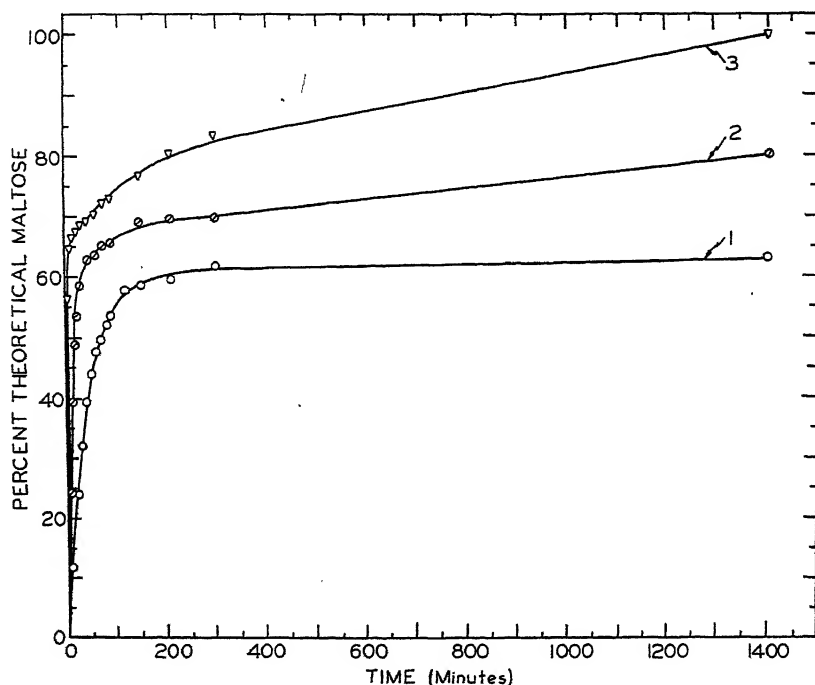


FIG. 1. Influence of concentration of amylase upon the extent of the hydrolysis of Lintner's soluble potato starch by purified maltase-free taka-amylase. Lintner's soluble potato starch 1 per cent, 0.05 M sodium chloride, 0.01 M acetate, pH 5.0. Hydrolyses at 40°. Amylase preparation per 1000 mg. of starch, Curve 1, 0.18 mg., Curve 2, 0.72 mg., Curve 3, 2.88 mg.

used. There is no evidence of a common limit in the hydrolysis far short of complete hydrolysis such as is observed with  $\beta$ -amylase from barley and from malted barley (5). These results are similar to those obtained with pancreatic amylase<sup>1</sup> and with malt  $\alpha$ -amylase (6-8).

The slowing down of the hydrolysis of starch by amylases has been ascribed in part to the inactivation of the amylase (9-11). The data summarized in Fig. 2 show that substrate added to a reaction mixture which

<sup>1</sup> Alfin, R., and Caldwell, M. L., unpublished work.

had reached the stage of very slow action (Curve 2) was hydrolyzed to practically the same extent (Curve 2a) as that reached in a comparable reaction mixture (Curve 1) which had contained initially the same ratio of amylase to substrate. Thus, under the conditions of these experiments, the stage of very slow action in the hydrolysis of starch by taka-amylase is not due to any appreciable inactivation of the amylase.

*Products Formed*—The products of the action of maltase-free highly purified taka-amylase on soluble potato starch were differentiated into glucose, maltose, and reducing dextrans by a modification of the method of

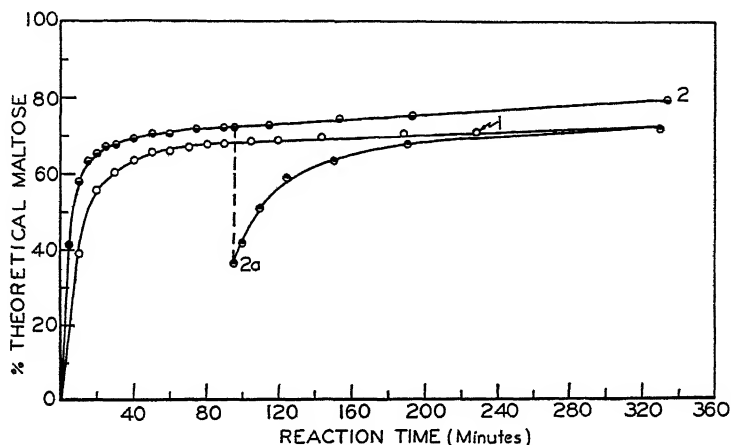


FIG. 2. Evidence that amylase had not been inactivated appreciably during the hydrolysis. Hydrolysis of additional substrate when this was added to a reaction mixture at the stage of very slow rate of change in the hydrolysis of starch by highly purified taka-amylase. Lintner's soluble potato starch 1 per cent, 0.05 M sodium chloride, 0.01 M acetate, pH 5.0. Amylase preparation per 1000 mg. of starch, Curve 1, 0.55 mg., Curve 2, 1.10 mg., Curve 2a, substrate added to part of reaction mixture of Curve 2 so as to bring the amylase concentration down to that in the reaction represented by Curve 1. Hydrolyses at 40°.

Somogyi (12) for the selective fermentation of glucose alone and of glucose and maltose together under different conditions by washed bakers' yeast. A study of the method showed that known concentrations of glucose and of maltose could be accounted for when added to reaction mixtures such as those used here and within the limits of concentration of these sugars encountered in the hydrolysis mixtures studied. Myrbäck (13, 14) has reported that certain trisaccharides are also fermented under the conditions used to ferment maltose. No attempt was made to distinguish between maltose and such sugars.

The data summarized in Table I show that maltose and glucose are formed in addition to reducing dextrans early in the hydrolysis of Lintner's

TABLE I

*Influence of Concentration of Amylase on Rate of Production of Maltose, Glucose, and "Reducing Dextrins" in Action of Purified Taka-amylase upon Lintner's Soluble Potato Starch*

Time	Reducing action calculated as maltose equivalents						
	Total	Reducing dextrins*		Maltose†		Glucose‡	
	Theory	Theory	Total	Theory	Total	Theory	Total

0.09 mg. amylase preparation per 1000 mg. starch‡							
min.	per cent	per cent	per cent	per cent	per cent	per cent	per cent
10	10.9	10.9	100	Not measurable		Not measurable	
20	18.9	18.6	98.4	0.3	1.6	"	
30	25.8	23.8	92.2	2.0	7.8	"	
40	31.5	27.7	87.9	3.7	11.7	0.1	0.3
50	36.9	31.1	84.3	5.4	14.7	0.4	1.0
60	40.7	32.9	80.8	7.3	17.9	0.5	1.2

0.18 mg. amylase preparation per 1000 mg. starch‡							
10	12	12.0	100	Not measurable		Not measurable	
20	24.1	21.7	90.0	1.6	6.7	0.8	3.3
30	32.1	28.1	87.4	3.2	10.0	0.8	2.5
40	39.4	32.2	81.6	5.6	14.3	1.6	4.1
50	44.2	34.6	78.2	6.4	14.5	3.2	7.3
60	47.5	35.4	74.5	9.7	20.4	2.4	5.2
70	49.8	31.9	64.0	14.7	29.6	3.2	6.4
80	52.3	31.4	60.0	16.9	32.3	4.0	7.7
90	53.8	31.3	58.2	18.5	34.3	4.0	7.5
120	57.9	31.3	54.1	23.3	40.3	3.2	5.6
150	58.6	29.7	50.7	24.1	41.1	4.8	8.2
210	59.5	28.9	48.6	26.6	44.6	4.0	6.8
300	61.8	28.9	46.7	26.5	42.9	6.4	10.4
1400	62.7	24.8	39.6	30.6	48.8	7.3	11.6

0.72 mg. amylase preparation per 1000 mg. starch‡							
5	24.1	21.7	90.0	1.6	6.7	0.8	3.4
10	39.4	30.5	77.5	7.3	18.4	1.6	4.1
15	49.0	30.5	62.2	15.3	31.2	3.2	6.6
20	53.8	31.3	58.2	19.3	35.8	3.2	6.0
25	58.6	29.7	50.7	24.1	41.1	4.8	8.2
30	58.6	29.8	50.7	25.6	43.7	3.2	5.5
45	62.7	26.5	42.3	31.4	50.0	4.8	7.7
60	63.5	25.8	40.5	32.9	51.8	4.8	7.6
75	65.2	24.9	38.3	34.6	53.0	5.7	8.7
90	65.9	25.0	37.8	35.2	53.5	5.7	8.6
150	69.2	25.0	36.1	36.9	53.3	7.3	10.6
210	69.9	24.8	35.5	37.8	54.0	7.3	10.5
300	69.9	24.8	35.5	37.8	54.0	7.3	10.5
1400	80.4	19.3	24.0	43.4	54.0	17.7	22.0

TABLE I—*Concluded*

Time	Reducing action calculated as maltose equivalents						
	Total	Reducing dextrins*		Maltose†		Glucose‡	
	Theory	Theory	Total	Theory	Total	Theory	Total
2.88 mg. amylase preparation per 1000 mg. starch‡							
min.	per cent	per cent	per cent	per cent	per cent	per cent	per cent
5	56.2	29.9	53.3	22.5	40.0	3.8	6.7
10	64.6	27.1	42.0	30.0	46.4	7.5	11.6
15	66.5	26.2	39.4	32.8	49.3	7.5	11.3
20	67.5	25.3	37.4	34.7	51.4	7.5	11.1
25	67.5	25.3	37.4	33.8	50.0	8.4	12.5
30	68.3	24.3	35.7	36.5	53.4	7.5	11.0
45	69.3	24.3	35.2	37.5	54.1	7.5	10.8
60	70.3	24.3	34.7	38.5	54.7	7.5	10.7
75	72.1	24.2	33.6	38.5	53.3	9.4	13.0
90	73.0	22.5	30.8	40.2	55.1	10.3	14.1
150	76.8	20.6	26.9	42.1	54.9	14.1	18.3
210	80.6	18.7	23.2	45.9	57.0	16.0	19.8
300	83.4	18.8	22.5	45.9	55.0	18.7	22.4
1400	100.0	9.2	9.2	68.4	68.4	22.4	22.4

\* Unfermented reducing products.

† Determined by selective fermentation with washed bakers' yeast (12).

‡ Lintner's soluble potato starch 1 per cent, 0.05 M sodium chloride, 0.01 M acetate, pH 5.0. Hydrolyses at 40°.

soluble potato starch by purified maltase-free taka-amylase. The rate at which these products appear depends upon the concentration of amylase.

The data summarized in Table II give average values for the distribution of the products formed at different stages in the hydrolysis of Lintner's soluble potato starch by highly purified maltase-free taka-amylase. The data show a rapid breakdown of starch to reducing dextrins of low average molecular weights and of relatively high reducing values. Thus, dextrins with an average degree of polymerization of 10 accounted for 99.5 per cent by weight of the total products when the hydrolysis mixture had reached the equivalent of 20 per cent of the theoretical maltose or when approximately 10 per cent of the glucose linkages of the starch had been ruptured. When the reducing value of the reaction mixture corresponded to 50 per cent of the theoretical maltose, reducing dextrins accounted for approximately 67 per cent of the total reducing value and for 85 per cent by weight of the products present.

These findings are in accord with the observations of many investigators in that the amylase of *Aspergillus oryzae* causes very rapid decrease in the viscosities of its substrates and very rapid disappearance of products which give color with iodine. In the present studies, a clear red color with iodine



was obtained when the reducing values of the reaction mixtures were equivalent to approximately 12 per cent of the theoretical maltose. The achromic stage was reached at approximately 20 to 25 per cent of the theoretical maltose.

After an average degree of polymerization of 10 had been reached, the average molecular weights of the reducing dextrins decreased more slowly. Dextrins with an average degree of polymerization of 4.3 accounted for

TABLE II  
*Products Formed from Lintner's Soluble Potato Starch by Purified Maltase-Free Taka-Amylase*

Reducing values, per cent of theoretical maltose			Glucose, † per cent of theoretical glucose	Dextrins ‡	
Total*	Reducing dextrins †	Maltose ‡		Per cent of total products by weight	Average degrees of polymerization §
10	10			100	20
15	15			100	13
20	19.5	0.5		99.5	10
25	23.2	1.5	0.15	98.3	8.5
30	26.9	2.5	0.30	97.2	7.2
35	30.0	4.0	0.50	95.5	6.4
40	32.4	6.0	0.80	93.2	5.7
50	33.6	13.5	1.45	85.0	5.1
60	29.3	26.0	2.35	71.6	4.9
70	23.6	38.0	4.2	57.8	4.9
80	19.5	44.5	8.0	47.5	4.9
90	15.1	54.5	10.2	35.3	4.7
100	9.5	68.5	11.0	20.5	4.3

\* Lintner's soluble potato starch, 1 per cent, 0.01 M acetate, 0.05 M sodium chloride, pH 5.0, 40° (3).

† Dextrins, calculated from reducing values after removal of glucose and maltose by fermentation (12).

‡ Determined by selective fermentation with washed bakers' yeast (12).

§  $\frac{\text{Weight of dextrins (mg.)}}{\text{Reducing values as maltose (mg.)}} \times 2$ .

20.5 per cent by weight of the products when the reducing value of the reaction mixture was equivalent to 100 per cent of the theoretical maltose.

Maltose and glucose could be detected by the method used (12) when the reaction mixtures had reached the equivalent of 20 and 25 per cent of the theoretical maltose respectively. These sugars were formed in increasing concentrations as the hydrolysis progressed and accounted for 33 per cent of the total reducing value when the hydrolysis had reached the equivalent of 50 per cent of the theoretical maltose and for 90 per cent of the total

reducing value when the equivalent of 100 per cent of the theoretical maltose had been reached. After the equivalent of approximately 50 per cent of the theoretical maltose had been reached, the concentrations of maltose and of glucose continued to increase, while that of the reducing dextrins decreased.

The concentration of glucose was much lower than that of maltose throughout the hydrolysis; there is no evidence that glucose continues to be formed after the production of maltose has ceased, as has been reported for unpurified preparations of certain amylases (15-17). In contrast to unpurified preparations of taka-diastrase, which contain maltase and possibly other glucosidases (16), the production of glucose is surprisingly low.

#### SUMMARY

A study of the action of highly purified taka-amylase has been made.

The preparations of taka-amylase showed no evidence of maltase activity.

The initial velocity and the extent of hydrolysis of starch by maltase-free taka-amylase depends within wide limits upon the concentration of amylase.

No evidence was found for a common limit in the extent of the hydrolysis of starch far short of complete hydrolysis such as is observed with  $\beta$ -amylase and as has been reported for this and certain other  $\alpha$ -amylases.

Under the conditions of these experiments, the slowing down of the hydrolyses was not due to any appreciable inactivation of the amylase.

Maltose, glucose, and reducing dextrins are all formed early in the hydrolysis of starch by maltase-free purified taka-amylase; in the later stages of the hydrolysis the concentrations of maltose and of glucose continue to increase, while that of the reducing dextrins decreases.

The concentration of glucose is much lower than that of maltose throughout the hydrolysis of starch by purified taka-amylase and there is no evidence that glucose continues to be formed after the production of maltose has ceased.

In contrast to unpurified preparations of taka-diastrase which contain maltase and possibly other glucosidases, the production of glucose from starch by purified taka-amylase is surprisingly low.

The average molecular weights and degrees of polymerization of the reducing dextrins decrease rapidly in the earlier stages of the hydrolysis of Lintner's soluble potato starch by purified taka-amylase; they decrease much more slowly in the later stages of the hydrolysis.

When 100 per cent theoretical "maltose" had been attained, the hydrolysis mixtures contained relatively high concentrations of maltose and glucose and relatively low concentrations of dextrins which approached tetrasaccharides in average molecular weight.

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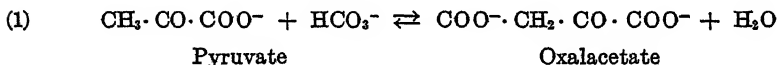
# THE EFFECTS OF TRIPHOSPHOPYRIDINE NUCLEOTIDE AND OF ADENOSINE TRIPHOSPHATE ON PIGEON LIVER OXALACETIC CARBOXYLASE\*

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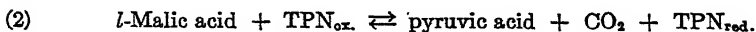
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The fixation of carbon dioxide by pigeon liver in the presence of added pyruvate was first described by Evans and Slotin (1) and the initial fixation mechanism was ascribed to the occurrence of the Wood-Werkman reaction; *i.e.*, to the addition of  $\text{CO}_2$  to pyruvate to form oxalacetate. Evidence supporting this point of view was afforded when Evans, Vennesland, and Slotin (2) succeeded in preparing from pigeon liver soluble protein preparations capable of fixing isotopic  $\text{CO}_2$  in a substrate mixture consisting of pyruvate, fumarate, malate, and lactate. An enzyme capable of decarboxylating oxalacetate and requiring  $\text{Mn}^{++}$  as a cofactor was shown to be present in these preparations. The initial fixation mechanism was attributed to the reversibility of this decarboxylation; *i.e.*, to the occurrence of reaction (1).



Wood, Vennesland, and Evans (3) found, however, that partially purified preparations of pigeon liver oxalacetate carboxylase showed no evidence for exchange of isotopic bicarbonate in the  $\beta$ -carboxyl carbon of oxalacetate such as would be expected if reaction (1) were reversible. Further clarification of this point was furnished by Utter and Wood (4), who found, in a detailed study of the exchange reaction, that adenosine triphosphate (ATP) had a decided stimulating effect.

Previously, Moulder, Vennesland, and Evans (5) had demonstrated that the pigeon liver preparations contained malic dehydrogenase which was active with both triphosphopyridine nucleotide (TPN) and diphosphopyridine nucleotide (DPN). Subsequently, Ochoa, Mehler, and Kornberg (6) obtained evidence for the occurrence in pigeon liver preparations of a reversible oxidative decarboxylation of malic acid, according to reaction (2).

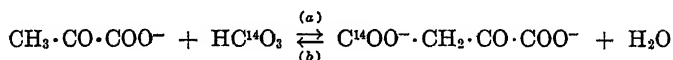



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$Mn^{++}$  was required as a cofactor, but ATP did not influence the reaction. These findings raise the question whether the primary fixation reaction occurs by way of reaction (1) or of reaction (2), or possibly by way of both reactions simultaneously. If reaction (2) constitutes the only initial channel whereby  $CO_2$  is fixed in the dicarboxylic acids, then the stimulating effect of ATP on the exchange of isotopic  $CO_2$  with the  $\beta$ -carboxyl carbon of oxalacetate might be explained in terms of the occurrence of TPN formation in the presence of ATP. Altman and Evans (7) have demonstrated such a synthesis of TPN when ATP alone is added to dialyzed pigeon liver extracts. Ochoa<sup>1</sup> has also reported that pigeon liver preparations can synthesize TPN from ATP and DPN.

Experiments described in this communication do not, however, support such an interpretation of the ATP effect. Direct examination of the exchange reaction,



revealed that, with partially purified preparations of pigeon liver oxalacetic carboxylase, added TPN gave little or no stimulation of the rate of the fixation reaction (a), whereas the stimulation by ATP, as reported by Utter and Wood (4), was invariably confirmed. On the other hand, examination of the effect of these substances on the decarboxylation reaction (b) revealed a marked stimulation by TPN, whereas ATP showed a small but definite inhibition.

### Methods and Materials

*Enzyme Preparation*—Crude extracts of pigeon liver were prepared by extraction of the acetone powder with water and dialysis against 0.025 M phosphate, pH 7.4, as previously described (2). The partially purified fractions were obtained in the course of purification studies. The essential fractionation steps employed for each preparation will be indicated in the text.

*Cofactors*—TPN of 78 per cent purity was prepared from beef liver by a modification<sup>2</sup> of the method of Warburg, Christian, and Griesse (8). DPN of 68 per cent purity was prepared by the method of Williamson and Green (9), followed by the series of salt fractionations up to the cuprous chloride stage, as described by Ohlmeyer (10). ATP was a gift from Dr. J. F. Speck of this department.

Oxalacetic acid was prepared according to Krampitz and Werkman (11).

<sup>1</sup> Ochoa, S., communication at the meeting of the Federation of American Societies for Experimental Biology, Chicago, 1947.

<sup>2</sup> Altman, K. I., unpublished method.

All manometric experiments were carried out with the standard Warburg manometric technique.

*Preparation and Measurement of  $C^{14}O_2$* —The  $C^{14}O_2$ , initially obtained as  $BaC^{14}O_3$ , was diluted approximately 100 times with inactive  $BaCO_3$ .  $C^{14}O_2$  was then liberated with HCl and absorbed in an appropriate volume of 0.1 N NaOH, the quantity of which was adjusted so that the final solution was about 0.05 N with respect to carbonates. This alkaline solution was kept as stock in a sealed container. 1 ml. was used in each experiment, and was neutralized with HCl to pH 7.4 in the presence of added phosphate buffer immediately before use. The  $C^{14}O_2$  from the medium of the experiments was collected in a measured volume of 1 N  $CO_2$ -free NaOH, by sucking a stream of  $CO_2$ -free air through the acidified solution and passing it through a sintered glass plate over which the alkali was placed. This procedure gave quantitative absorption of the  $CO_2$ . The  $C^{14}O_2$  liberated by aniline citrate was similarly collected. Aliquots of the alkali samples containing the  $C^{14}O_2$  were analyzed for total carbonate content by the standard Warburg manometric technique. The  $C^{14}$  was then precipitated from the remainder of the solution by the addition of  $BaCl_2$ . The barium carbonate was centrifuged and washed with water by repeated resuspensions and centrifugations until the wash water was free from alkali. The precipitate was then washed once with ethanol, suspended in a minimum volume of ethanol, transferred to a tared aluminum cup with a flat round bottom of 3.5 sq. cm. area, and dried to constant weight. Counting and corrections for self-absorption were carried out according to Reid (12). All figures given are thick sample counts corrected for background. Checks on known dilutions indicated that the procedure employed gave reproducibility and correct proportionality within 5 per cent.

*Procedure of Exchange Experiments*—The "exchange" experiments were designed to determine the extent to which  $C^{14}O_2$  entered the  $\beta$ -carboxyl group of oxalacetate during decarboxylation of the latter by the pigeon liver enzyme. The procedure employed was a minor modification of methods previously described (3, 4, 13). The essential steps are, first, the incubation of the enzyme with oxalacetate in the presence of  $HC^{14}O_3$ , then the removal of all  $C^{14}$  present as carbonates, and finally the liberation of the  $\beta$ -carboxyl carbon of the oxalacetate.

In the experiments described in this paper, the decarboxylation was continued to about two-thirds completion. The pH was initially 6.7, but became more alkaline as the reaction progressed. Incubation was carried out at 30° in stoppered test-tubes without shaking. The reaction was stopped by introducing the mixture through a separatory funnel into 5.0 ml. of ice-cold 10 per cent metaphosphoric acid plus a drop of caprylic alcohol. The mixture was kept in an ice bath until the addition of aniline

citrate. The  $C^{14}O_2$  liberated from the medium by the addition of acid was completely removed by flushing the solution with  $CO_2$ -free air. The air stream was passed through alkali for quantitative absorption of the  $C^{14}O_2$ , as described in the previous section. The last traces of  $C^{14}O_2$  which might be present in the solution were then rinsed out by 10 minutes rapid flushing with  $CO_2$  gas. The  $CO_2$  remaining from the rinse was removed quantitatively by flushing with  $CO_2$ -free air. A sample was collected for analysis of radioactivity. In all experiments reported, the rinse samples were found to contain no detectable radioactivity, indicating adequate removal of the  $C^{14}O_2$  of the medium. The  $\beta$ -carboxyl carbon of oxalacetic acid was liberated by the addition of 5 ml. of aniline citrate, and the  $CO_2$  was collected quantitatively and analyzed as previously described.

*Method of Assaying Oxalacetic Carboxylase*—In order to characterize the enzyme preparations employed in these studies, quantitative assays of decarboxylation activities were made. A standard test system was adopted for the assays. The enzyme activity was determined by manometric measurement of the rate of carbon dioxide evolution from oxalacetate in 0.1 M acetate buffer, pH 5.0, in the presence of 0.001 M  $MnCl_2$ . 1 mg. of oxalacetic acid is tipped from the side arm at zero time. The total volume is 2.0 ml. The rate of decarboxylation of oxalacetate after heat inactivation of the enzyme (1 minute in a boiling water bath) must be subtracted in order to obtain a figure which is proportional to the amount of the enzyme used. The results are then expressed in terms of carbon dioxide evolution, as microliters per minute per ml., or as microliters per minute per mg. of N. The latter figure is used as a measure of the purity of the enzyme. Since TPN has been found to have a stimulating effect on the rate of decarboxylation, as will be described, assays are run both in the presence and in the absence of this cofactor.

Justification for the use of the assay system described rests on a demonstration of direct proportionality between enzyme concentration and activity. Unless the procedure is used with a number of precautions, proportionality may not be obtained. The assay is valid only when the reaction observed is zero order; *i.e.*, when microliters of  $CO_2$  evolved, plotted against time, give a straight line. Such is not always the case. Fresh, undialyzed extracts of pigeon liver acetone powder, for example, never give a zero order reaction. However, the reaction almost always becomes zero order (until about two-thirds completion) after dialysis against 0.025 M phosphate, pH 7.4, or 0.025 M veronal, pH 7.8. The enzyme may also be dialyzed against water, but loss of activity is more likely to occur in the unbuffered medium.

Fig. 1 shows the type of data obtained in a representative experiment designed to test proportionality. Figures obtained during the initial part

of the test, before deviations from linearity occurred, are given. The 2 minute reading is taken as the initial point, to avoid errors in timing associated with tipping. The data in Fig. 1 indicate that the blank value, which was the same for all samples used, must be subtracted in order to obtain proportionality. This fact alone, apart from any theoretical considerations, justifies the subtraction of the blank. The data also show the

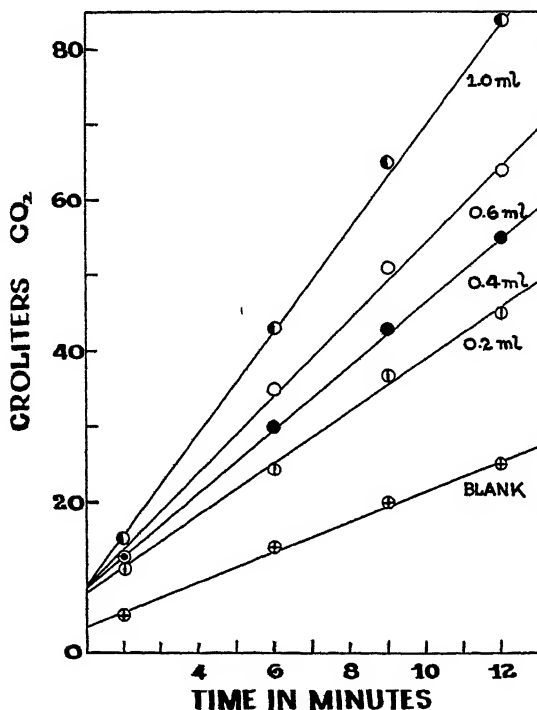


Fig. 1. Proportionality of rate of  $\text{CO}_2$  evolution to quantity of pigeon liver oxalacetic carboxylase. Partially purified enzyme containing 80  $\gamma$  of N per ml. Conditions those of the standard test system with 50  $\gamma$  of TPN added per vessel. This amount of TPN gave 140 per cent stimulation of the reaction rate.

deviation from proportionality which is usually obtained when the actual rate measured becomes lower than 2 microliters per minute after subtracting the blank. Thus, the observed rates, expressed as microliters per minute per ml., calculated after subtracting the blank, are 5.0, 5.5, 5.4, and 7.9 for 1.0, 0.6, 0.4, and 0.2 ml., respectively.

In this experiment, a purified enzyme prepared as described in Table I, Enzyme B, was used. The heated enzyme had no effect on the blank, which was equal in each case to the rate of decarboxylation observed in the test system in the absence of enzyme. With unpurified preparations this



is not always the case. The heat-inactivated protein may either accelerate or inhibit the catalysis due to  $Mn^{++}$  alone.

The blank reaction is never zero order, but always first order. This becomes apparent when it is followed over a longer period of time than shown in Fig. 1. The subtraction of the blank, as indicated, does not constitute an ideal correction. With high rates of enzyme activity, the blank correction as applied is too large. It would be more accurate to calculate the correction figure from the observed first order reaction constant of the

TABLE I  
*Effect of Varying TPN Concentrations on Activity of Oxalacetic Carboxylase from Pigeon Liver*

Enzyme	TPN added	Rate of decarboxylation calculated relative to rate without TPN taken as 100
	<i><math>\gamma</math> per 2 ml.</i>	
A. Dialyzed, aged extract of pigeon liver acetone powder; activity without TPN = 15 microliters per min. per ml.	0	100
	0.65	107
	3.25	120
	6.5	135
	32.5	146
	65	153
B. *Partially purified oxalacetic carboxylase; activity without TPN = 32 microliters per min. per mg. N	0	100
	17	218
	51	239
	170	244
	510	235

The conditions used were those of the standard test system; 0.1 M acetate buffer, pH 5.0, 0.001 M  $MnCl_2$ ; total volume of test system 2.0 ml.; 1 mg. of oxalacetic acid tipped in from side arm to start reaction; incubated at 30°; decarboxylation rates corrected for blanks; TPN has no effect on blanks.

\* Prepared by a procedure, the essential steps of which involve fractional precipitation with  $(NH_4)_2SO_4$  (fraction precipitating between one-third and two-thirds saturation), absorption on  $Ca_3(PO_4)_2$  gel at pH 4.3, and elution at pH 7.8, and precipitation with nucleic acid which is removed with salmine.

blank and the average amount of oxalacetate actually remaining during the period of observation of the enzyme reaction. For routine purposes, however, the added accuracy so achieved does not seem sufficient to warrant such a calculation.

The conditions of the test system are not optimal with respect to buffer concentration and pH. Higher activity is obtained at higher pH values and lower buffer concentrations. It was thought desirable, however, to keep the buffer capacity of the test system relatively high in order to preclude the possibility that reaction kinetics might be influenced by pH changes occurring as a result of the decarboxylation.

The manganese concentration used in the test system is optimal, as indicated in Fig. 2, which shows the variation of activity with  $Mn^{++}$ . Although rates of decarboxylation uncorrected for the blank show a steady increase with rise in  $Mn^{++}$ , the enzyme activity calculated after correction for the blank indicates the existence of an optimum at about 0.001 M, below and above which activity declines.

Though 5 per cent  $CO_2$ , 95 per cent  $N_2$  is preferable as a gas phase, assays may be run in air for convenience. In this case, however, the possibility of the occurrence of oxidative side reactions must be guarded against.

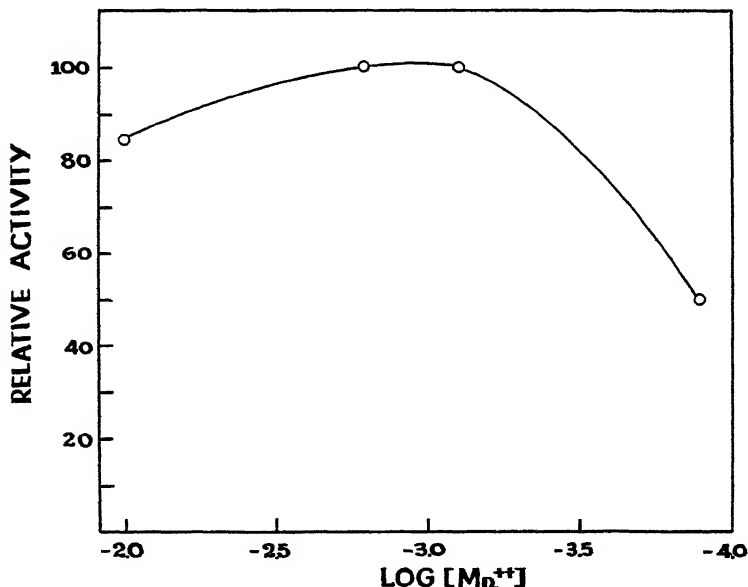


Fig. 2. Effect of manganese concentration on activity of pigeon liver oxalacetic carboxylase. Enzyme and conditions as in proportionality experiment. 0.5 ml. of enzyme and 17  $\gamma$  of TPN present in each vessel.

Oxidation of added oxalacetate is seldom observed with pigeon liver acetone powder extracts, but may occur to a small but significant extent in some fractions. It can probably be explained in terms of the type of oxidative decarboxylation catalyzed by myoglobin and  $Mn^{++}$  (14, 15). It is customary in this laboratory to use air as a gas phase, and to determine the amount of oxalacetate added to the test system by decarboxylating an aliquot with an ion such as  $Ni^{++}$ ,  $Co^{++}$ , or  $Al^{+++}$ . The  $CO_2$  evolved when the test system is run to completion should agree within 5 per cent with the assay of the amount of substrate added. Otherwise reactions other than a simple non-oxidative decarboxylation must be suspected.

The determination of the amount of oxalacetate actually added at the time of tipping is simpler than weighing  $1.00 \pm 0.05$  mg. for each sample. About 50 per cent deviation in the amount of substrate actually used will not influence the results, provided the blank contains the same amount as the test with the active enzyme.

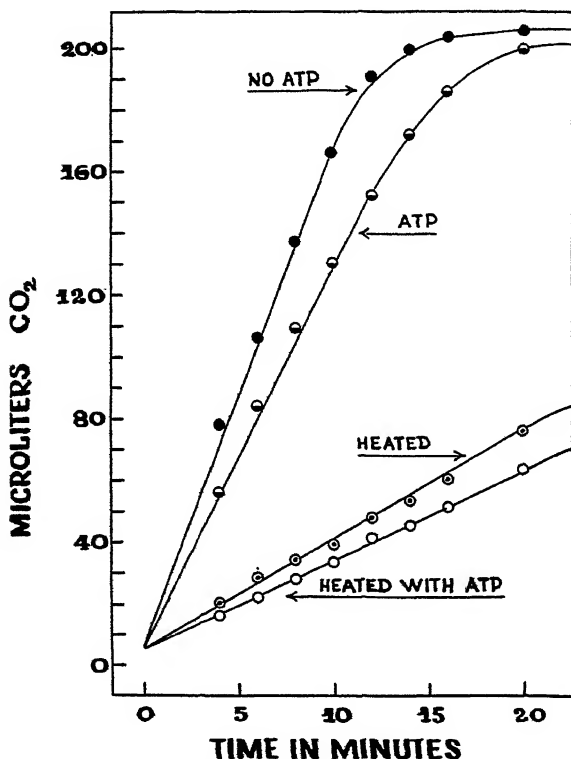


FIG. 3. Effect of ATP on oxalacetic carboxylase from pigeon liver. Conditions those of standard test system. 1.2 micromoles of ATP added to 2 ml. Enzyme used was 0.5 ml. of dialyzed pigeon liver acetone powder extract.

### Results

*Effects of ATP and of TPN on Decarboxylation of Oxalacetate*—When the action of ATP and of TPN on the enzymic decarboxylation of oxalacetate was measured under the conditions of the standard test system, *i.e.* 0.1 M acetate, pH 5.0, and 0.001 M  $MnCl_2$ , it was found that ATP caused an inhibition and that TPN caused an acceleration.

A typical experiment with ATP is shown in Fig. 3. It can be seen that the inhibition effect is not large; indeed, the action on the blank is often almost as great proportionally as the action on the active enzyme. It was

necessary, in experiments of this type, to control carefully the effect of salts present in the ATP solution, since oxalacetic carboxylase is non-specifically inhibited by salt.

In contrast to the small inhibitory effect of ATP, the stimulating action of TPN was found to be of considerable magnitude under some circumstances. Of close to 50 different preparations tested, all were found to show some stimulation by TPN. This was true of undialyzed fresh extracts of

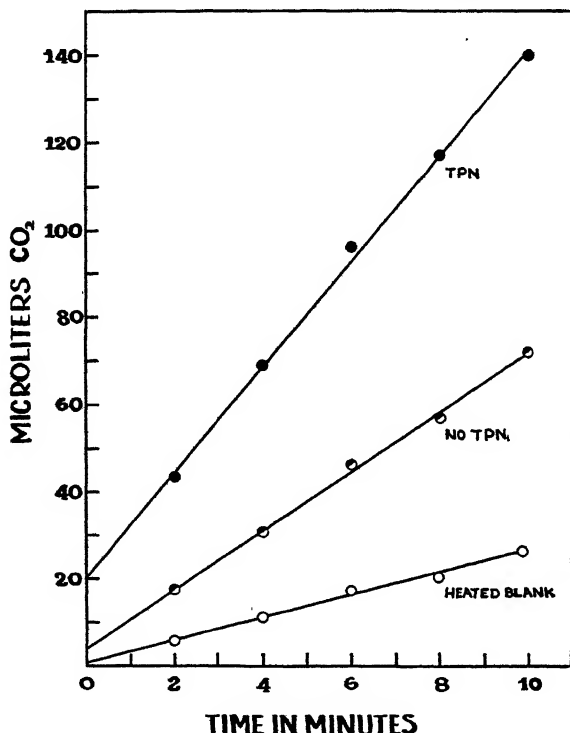


Fig. 4. Effect of TPN on oxalacetic carboxylase from pigeon liver. Conditions those of standard test system. 17  $\gamma$  of TPN added in 2.0 ml. of TPN had no effect on the heated blank. Enzyme used was 0.3 ml. of solution used in Series 2, Table II.

acetone powder, though the stimulation here was only of the order of 10 to 20 per cent, and may reflect the fact that the extract is diluted in the test system. Aged, dialyzed extracts showed stimulations of the order of 30 to 50 per cent, and in some partially purified preparations stimulations of as great as 200 per cent were noted. It has not yet been possible to obtain enzyme preparations active only in the presence of added TPN and inactive in its absence.

Fig. 4 shows data obtained in a test of the effect of TPN on the enzyme

preparations used in the experiments of Series 2, Table II. These are data from an actual assay. It can be seen that the stimulation does not alter the zero order kinetics observed during the initial part of the reaction. The amount of TPN used, 17  $\gamma$  per 2 ml., was sufficient to give 90 per cent of the maximum effect obtainable. (To conserve TPN, routine assays were made with 17 to 20  $\gamma$  of TPN per 2 ml.) This is indicated by the data in Table I, which show the relative effects of varying amounts of TPN

TABLE II  
*Effect of ATP and TPN on  $C^{14}O_2$  Fixation in Oxalacetate*

Series No.	Cofactors added	Bicarbonate of medium at end of reaction		$\beta$ -Carboxyl carbon of oxalacetic acid		
		Amount	Radio-activity	Amount	Radioactivity	
		micro-liters	counts per min.	micro-liters	counts per min.	per cent bicarbonate activity
1	None	5080	1925	2460	$38.8 \pm 1.3$	2.02
	1 micromole TPN	4000	2184	3460	$12.6 \pm 0.8$	0.58
	1 " ATP	5640	1642	3220	$90.0 \pm 2.2$	5.48
2	None	5440	1969	2920	$3.3 \pm 0.5$	0.17
	1 micromole TPN	4360	2005	3760	$3.1 \pm 0.5$	0.15
	1 " ATP	5920	1740	2720	$64.0 \pm 1.3$	3.68

Composition of initial reaction mixture:  $2.4 \times 10^{-3}$  M  $MnCl_2$ , 0.03 M potassium phosphate, pH 6.7, 0.03 M Na oxalacetate. About 1000 microliters of  $NaHC^{14}O_3$  were added to a total volume of 8.2 ml. 4 ml. of enzyme were added in each case. In Series 1, the enzyme solution had an activity in the standard test system of 14 microliters per minute per ml. in the absence of added TPN, and 30 microliters per minute per ml. in the presence of TPN (9  $\gamma$  per ml.). The activity of the enzyme solution used in Series 2 was 10 microliters per minute per ml. in the absence of TPN and 26 microliters per minute with 9  $\gamma$  of TPN per ml. Incubation was carried out in stoppered tubes at 30° for 30 minutes in Series 1, and for 35 minutes in Series 2.

tested on two different preparations. One of these, an aged, dialyzed extract, gave a maximum stimulation of 53 per cent; the other, a partially purified preparation, gave a maximum stimulation of 135 per cent.

When DPN was tested in amounts comparable to those employed with TPN, no measurable effect was obtained. The addition of DPN with ATP to the test system likewise gave no effect which could not be caused by the ATP alone.

It should be noted that the effects described in this section apply to those of the standard test system at pH 5.0. Detailed data on the enzymic decarboxylation at pH close to neutrality have not been obtained, and the nature of the kinetics of the reaction at such pH is not known. A few at-

tempts to demonstrate effects of ATP and TPN on the rate of decarboxylation at pH 6.7 have given no evidence for effects similar to those observed at pH 5.0.

*Effects of ATP and of TPN on Fixation of  $C^{14}O_2$  in  $\beta$ -Carboxyl Carbon of Oxalacetic Acid*—Tests of the exchange of  $C^{14}O_2$  with the  $\beta$ -carboxyl carbon of oxalacetate as catalyzed by dialyzed but unpurified extracts of pigeon liver acetone powder confirmed the findings of Utter and Wood (4). There was considerable variation among different extracts in the amount of exchange observed in the absence of added cofactors other than  $Mn^{++}$ , but the addition of ATP always gave a marked increase in the magnitude of the reaction. The addition of TPN to such extracts gave somewhat variable results, showing effects similar to those observed by Utter and Wood (4) with DPN.

It was thought that more significant results might be obtained with partially purified preparations of the enzyme, since it seemed possible that the stimulation of fixation by either ATP or TPN might be eliminated on purification. When such experiments were conducted, it was found that the stimulating effect of TPN was eliminated completely, whereas that of ATP became more marked. The results are shown in Table II. Two sets of experiments were run. In Series 1 the enzyme solution was prepared by fractional precipitation with  $(NH_4)_2SO_4$ , followed by an adsorption and elution from calcium phosphate gel. The enzyme solution used in Series 2 was prepared from the solution employed in Series 1 by precipitation with nucleic acid which was removed with salmine. This step caused little change in purity, but removed some pigmented material. The data show that there was considerable fixation of  $C^{14}O_2$  in the absence of added cofactors in Series 1. TPN inhibited this fixation about 71 per cent. ATP gave 170 per cent stimulation. In Series 2, where fixation in the absence of added cofactor was very low, TPN had no effect, whereas ATP gave 2000 per cent stimulation.

#### DISCUSSION

Reactions (1) and (2) represent alternate mechanisms for the initial fixation of  $CO_2$  in the dicarboxylic acids. It is possible that both may operate independently or that either alone may constitute the initial reaction. The reversibility of reaction (1) is dependent on ATP, whereas reaction (2) is dependent on TPN. This might indicate an independence of the two reactions. Several facts, however, point to the existence of an intimate relationship; namely, the close association of the enzymes catalyzing the two reactions, as reported by Ochoa,<sup>1</sup> and also the effect of TPN on the decarboxylation of oxalacetic acid. This latter effect may, in fact, be considered unusual unless the decarboxylation involves an oxidation-reduction.

The results reported in this paper do not provide a final solution to the problem of the detailed mechanisms involved. They do show, however, that it is not possible at present to interpret the effect of ATP on CO<sub>2</sub> fixation in oxalacetate in terms of the ability of ATP to cause TPN formation. Though the possibility exists that impurities in the TPN preparation may account for the stimulation of the decarboxylation, it is difficult to see how such impurities could invalidate the above conclusion.

#### SUMMARY

The effects of ATP and TPN on the decarboxylation of oxalacetic acid by oxalacetic carboxylase from pigeon liver have been examined. ATP causes a small inhibition, and TPN gives a marked stimulation.

On the other hand, the exchange reaction between C<sup>14</sup>O<sub>2</sub> and the  $\beta$ -carboxyl carbon atom of oxalacetic acid is stimulated by ATP and not by TPN.

The significance of these findings in relation to the mechanism of CO<sub>2</sub> fixation in dicarboxylic acids is discussed.

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# REDUCTION OF IRON BY FOODS IN ARTIFICIAL GASTRIC DIGESTION\*

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In order to carry out studies on the reduction of iron in the gastrointestinal tract a method was necessary for the determination of both ferric and ferrous forms of iron in digestion mixtures. The excellent methods existing for the determination of total iron in foods and biological materials (1) did not meet this requirement. Attempts to use the dipyridyl color reaction to determine ferrous iron in the presence of ferric iron were not successful. Thompson (2) has presented a thiocyanate procedure for total iron in which the colored complex is extracted with isobutanol. By adding the oxidizing agent to one aliquot and omitting it in another and by extracting with a mixture of isobutanol and tertiary butanol a satisfactory modification was developed.

## EXPERIMENTAL

It was found that in a solution of ferrous iron, in the absence of ferric iron, a color developed slowly with thiocyanate after the addition of isobutanol. The iron solution used was a solution of ferric chloride which had been run through a Jones reductor. Aliquots of this were tested with dipyridyl without the addition of a reducing agent and the color read at once. The results showed that the iron was 98 to 99 per cent in the ferrous form. When aliquots were treated with thiocyanate, no color developed in the absence of isobutanol for at least 2 minutes. After the addition of this alcohol, a color began to develop at the zone of contact. The presence of impurities in the isobutanol was ruled out because the oxidation of the ferrous iron occurred even if the alcohol was purified by repeated fractional distillation or recrystallization. Treatment of the alcohol with various oxidizing and reducing agents in acid and alkaline solution did not prevent oxidation of the iron. Other alcohols commonly used as selective solvents gave the same results with the exception of tertiary butanol. The latter

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is miscible with water. However, when tertiary butanol is mixed with a definite quantity of isobutanol, two layers can be obtained and the oxidation of the iron prevented. It was imperative that 10 ml. of 20 per cent potassium thiocyanate solution be used, not only to produce the color but to act as a salting-out agent for the alcohols.

The method as modified has been applied to the determination of soluble iron in various foods and biological materials.

### *Procedure*

Appropriate aliquots (preferably containing from 10 to 40  $\gamma$  of ferric or total iron) are transferred to two 125 ml. separatory funnels, and distilled water is added to a total volume of 25 ml. Exactly 5 ml. of concentrated HCl and 20 ml. of tertiary butanol are added. To the mixture that

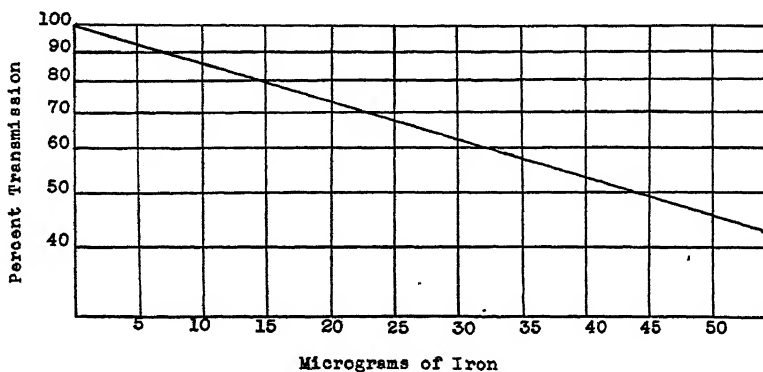


FIG. 1. Typical standard curve for the determination of iron by the modified thiocyanate procedure.

is to be tested for total iron ( $\text{Fe}^{++}$  plus  $\text{Fe}^{+++}$ ) 2 ml. of a 4 per cent potassium persulfate solution are added, while to the one that is to be assayed for ferric iron only 2 ml. of distilled water are added. 10 ml. of 20 per cent potassium thiocyanate solution are added to develop the color. This is followed immediately by the addition of 15 ml. of isobutanol. The samples are shaken for 30 seconds, and then allowed to stand until two layers are formed. The lower layer is then discarded and the alcoholic layer transferred to a dry 50 ml. Erlenmeyer flask. Prior to reading the per cent of transmission, the last traces of water are removed by the use of a small amount of anhydrous sodium sulfate. Should the solutions remain cloudy, they may be centrifuged. The per cent transmission should be read immediately.

The readings are taken at 485  $m\mu$  with the reagent blanks set at 100 per cent transmission. The micrograms of iron are obtained from a standard

curve. Fig. 1 represents a typical curve. Ferrous iron is the difference between the total iron and the ferric iron.

Two reagent blanks are used: one for samples containing potassium persulfate, the other for samples without the persulfate. The transmission of the two blanks should not differ by more than 4 per cent.

*Reagents—*

Iron-free distilled water.

Concentrated hydrochloric acid; minimum sp. gr. 1.1875.

Tertiary butanol; Eastman Kodak Company, No. 820; redistilled.

Isobutanol; Eastman Kodak Company, No. 303; redistilled.

Potassium persulfate; reagent grade; 4 per cent solution in iron-free distilled water. This solution should be prepared at frequent intervals (weekly).

Potassium thiocyanate; 20 per cent solution in iron-free distilled water.

Anhydrous sodium sulfate; reagent grade, Mallinckrodt.

Standard iron solution; stock solution. Dissolve iron wire of known iron content in a minimum amount of 20 per cent HCl to which 2 ml. of concentrated nitric acid have been added. Evaporate the acid solution to dryness, and redissolve the residue in the minimum amount of HCl and dilute the solution with iron-free distilled water so as to give a concentration of 1 mg. of iron per ml. To prepare a freshly oxidized standard iron solution treat 10 ml. of the stock solution with 2 ml. of 30 per cent hydrogen peroxide and 2 ml. of concentrated ammonium hydroxide. Concentrate this mixture to about 5 ml. The precipitate is dissolved by the addition of 2 ml. of concentrated HCl and 30 ml. of iron-free distilled water. This is transferred to a 100 ml. volumetric flask and the final volume is adjusted with the iron-free distilled water.

Celite 501; Johns-Manville. This is used as a filter aid. Since Celite 501 contains considerable iron, the latter is removed by washing with 10 per cent HCl until iron-free. The excess acid is removed by washing with iron-free distilled water and the Celite is then dried.

*Reliability of Method*

Table I represents a number of typical results obtained with our modified method as applied to pure solutions of ferrous iron and mixtures of ferrous and ferric iron. The ferrous iron added was obtained from an iron solution run through a Jones reductor, while the ferric iron was treated with hydrogen peroxide to insure complete oxidation. The samples containing the potassium persulfate were read against the blank to which the oxidizing agent was added, while those without the persulfate were read against the blank from which the oxidizing agent was omitted. The results obtained

are within the limits of experimental error. With an increase in the concentration of iron the per cent of error decreases.

*Reduction of Iron by Foods in Artificial Gastric Digestion*

Food samples in the quantities indicated below were incubated at 37.5° for 3 hours with either hydrochloric acid (pH  $1 \pm 0.2$ ) or with a 0.5 per cent solution of commercial pepsin (1:10,000) in hydrochloric acid (pH  $1 \pm 0.2$ ). The desired quantity of each food was mixed with 90 ml. of the

TABLE I  
*Recovery of Ferrous Iron Alone or in Presence of Ferric Iron*

Sample No.	Ferrous iron added	Ferric iron added	Ferric iron found		Ferrous iron found
			No $K_2S_2O_8$ added	$K_2S_2O_8$ added	
	$\gamma$	$\gamma$	$\gamma$	$\gamma$	$\gamma$
1	10	0	0.5	10.0	9.5
2	10	0	0.3	9.9	9.6
3	20	0	0.5	19.8	19.3
4	20	0	1.5	20.0	18.5
5	30	0	1.8	29.7	27.9
6	30	0	1.4	30.0	28.6
7	40	0	1.3	39.8	38.5
8	40	0	1.4	39.8	38.4
9	10	10	11.3	20.5	9.2
10	10	10	11.2	20.1	8.9
11	20	20	21.5	40.2	18.7
12	20	20	21.4	39.9	18.5
13	25	25	26.3	50.4	24.1
14	25	25	26.2	50.2	24.0
15	30	30	31.0	60.6	29.6
16	30	30	31.4	60.5	29.1

hydrochloric acid or pepsin-hydrochloric acid and 10 ml. of a freshly oxidized iron solution, so that each ml. of the reaction mixture represented 10  $\gamma$  of ferric iron. In the control samples 10 ml. of iron-free distilled water were used in place of the iron solution.

The quantities of foods used per 100 ml. of reaction mixture were as follows: air-dried whole wheat bread 7 gm., air-dried white bread (not enriched) 7 gm., acetone-dried beef muscle 7 gm., cooked egg yolk 7 gm., cooked egg white 7 gm., cooked beef (fat-free) 12.5 gm., boiled white potatoes 12.0 gm., peas (canned) 12.0 gm., raw cabbage 12.0 gm. For homogenized milk (acidified to pH 1), orange juice, and tomatoes (minced in a Waring blender) 50.0 ml. portions were mixed with 10 ml. of iron-free distilled water or 10 ml. of iron solution. The total volume was adjusted

to 100 ml., with the hydrochloric acid solution or the acid pepsin solution. After the 3 hour incubation period the reaction mixtures were filtered through a column of Celite contained in a carbon tube. The filtration process was found not to change the quantity or the state of oxidation of the soluble iron of solutions subjected to it.

Oxidation-reduction potentials and pH values of mixtures were determined at the beginning and end of the digestion periods. The final values are given in Table II. The  $E_h$  determinations were made with a bright platinum electrode and potentiometer. Potentials are expressed as millivolts. The sign is in accord with the European convention commonly used by biochemists and is referred to the standard hydrogen electrode. According to the same nomenclature, the oxidation-reduction potential of the  $\text{Fe}^{+++}$ - $\text{Fe}^{++}$  system is +747. All of the food mixtures studied by us have potentials less positive than this, and so some reduction of added ferric ions would be expected in all cases. This was found to be true except with egg yolk, in which instance the iron was completely or almost completely bound. In other cases, also, complex formation has limited the amount of reduction. It must be borne in mind also that the metal ions may form complexes not only with organic dietary constituents but with  $\text{Cl}^-$  and other inorganic ions. No marked parallelism of  $E_h$  and reduction was therefore found or was to be expected. Foods highest in iron-reducing value, as oranges and tomatoes, did, however, show the greater reducing tendency as measured by  $E_h$  values. Further knowledge of the state of the iron in the mixtures will be needed to correlate potentials with iron reduction more closely.

That the hydrochloric acid of the reagents used did not in the presence of foods give rise to any oxidizing or reducing agents influencing the results was shown by experiments in which such foods when incubated with hydrochloric acid or pepsin-hydrochloric acid for 3 hours gave the same results for ferric and ferrous iron whether the analysis was completed immediately after addition of the reagent hydrochloric acid or half an hour later. The use of actinic ray flasks to protect from sunlight was found to be unnecessary.

Table II shows the results obtained with the various foods. Each sample was incubated with and without added iron (control). The results expressed in Table II are derived in the following manner:  $a$  = total iron found in the control;  $a'$  = ferrous iron found in the control;  $b$  = total iron found in the sample with added iron;  $b'$  = ferrous iron found in the sample with added iron;  $c$  = 50  $\gamma$  of ferric iron per 5 ml. of reaction mixture; per cent reduction =  $[(b' - a')/c] \times 100$ ; per cent complex formation =  $[(c - (b - a))/c] \times 100$ .

As is indicated in the formula, by per cent complex formation is meant

that per cent of iron added to the food mixtures that is present in such a form as not to react with the thiocyanate under the conditions of the determination. Iron is known to form complexes with proteins and many other substances.

TABLE II

*Reduction and Complex Formation of Iron by Foods on Incubation with chloric Acid and Pepsin-Hydrochloric Acid Solutions*

$E_h$  and pH values for the mixtures at the end of the incubation periods.

Food	Hydrochloric acid				Pepsin-hydrochloric acid			
	Reduction	Complex formation	$E_h$	pH	Reduction	Complex formation	$E_h$	pH
	per cent	per cent	mv.		per cent	per cent	mv.	
Whole wheat bread.....	9.0	62.0	+515	1.29	23.0	54.0	+496	1.39
White bread.....	17.7	43.5	+541	0.98	43.5	7.0	+533	0.98
Dried beef muscle.....	39.6	58.0	+447	1.90	31.0	57.0	+330	3.09
Cooked beef.....	10.5	11.5	+444	1.49	28.0	18.0	+486	1.56
“ egg yolk.....	0.0	92.5	+497	1.06	0.0	100.0	+488	1.28
“ “ white.....	20.3	6.0	+390	1.23	29.0	6.0	+423	1.34
Homogenized milk.....	20.0	70.5	+480	1.26	11.5	21.0	+471	1.20
Orange juice.....	78.0	9.0	+402	1.78	77.0	16.5	+375	1.80
Tomatoes.....	86.5	10.0	+378	1.70	98.0	5.0	+395	1.90
Boiled white potatoes.....	54.5	15.0	+518	1.24	39.0	23.5	+471	1.45
Canned peas.....	22.0	67.5	+508	1.09	4.5	89.5	+536	0.99
Raw cabbage.....	81.5	10.3			62.0	17.0		

## DISCUSSION

From the results in Tables I and II it will be seen that by the method proposed soluble ionizable ferric and ferrous iron could be determined in the presence of each other in pure solutions as well as in artificial gastric digestion mixtures. The results on foods are averages of at least two experiments in each case. The slight reduction due to the pepsin preparation did not appear to influence the results significantly, since in a number of cases reductions were less in the presence than in the absence of pepsin.

No conclusions can at present be drawn from differences in degrees of iron reduction obtained on treatment of foods with hydrochloric acid as compared with pepsin-hydrochloric acid. While white bread and cooked beef showed greater reductions on the pepsin-hydrochloric acid treatment, other foods, such as egg white, did not. Studies on individual components of foods should throw more light on this matter.

With regard to complex formation, it was found that in general whenever the degree of reduction was rather small the per cent of complex formation

was high. A striking example of this can be seen in the case of egg yolk in which there was no reduction but 100 per cent complex formation. Tompsett (3) in his experiments reported that iron was not reduced by egg yolk. With egg albumin, on the other hand, we have very little complex formation but a relatively high per cent reduction. The recovery of added soluble iron is related to the amount of iron entering into complex formation with the particular foods.

Ferric iron was reduced by all of the foods tested except egg yolk. Fresh fruits and vegetables (oranges, tomatoes, cabbage) gave the highest values (up to 98 per cent). Considerable reductions were also noted with breads, meats, and egg white. Milk had a lesser effect. It is clear that a very significant amount of reduction of iron may occur with foods under conditions of artificial gastric digestion.

Similar experiments were carried out on pure sugars, the following amounts being added to hydrochloric acid solutions with a final volume of 100 ml.: glucose 0.337 gm., fructose 0.337 gm., maltose 0.607 gm., dextrin 1.685 gm., lactose 0.545 gm. The percentage reductions of added iron were for glucose 11.0, fructose 11.0, maltose 12.0, dextrin 9.0, and lactose 15. The results with lactose were confirmed on a highly purified preparation. Very similar results were, however, obtained with hydrochloric acid solutions incubated under similar conditions but in the absence of sugar, reductions of 10 to 11 per cent being noted and with pepsin-hydrochloric acid 12 per cent. These reductions took place gradually, over the 3 hour period, and indicate that any reducing effect of the sugars must be very slight.

Ascorbic acid in 25 mg. quantities (similar to the amounts present in the tomato experiments) gave 83 per cent reduction, compared to 86.5 per cent noted with tomatoes. Ascorbic acid is probably responsible therefor, for the high reduction values with tomatoes as well as with orange juice, and may be partly responsible for the fairly high results with potatoes, since these contain appreciable amounts of ascorbic acid.

That proteins and protein digestion products may have reducing effects is suggested by the results with high protein foods and is confirmed by some experiments with lactalbumin. A water-soluble lactalbumin preparation in amounts of 7 gm. gave reductions in hydrochloric acid solution of 21.4 per cent, indicating that this protein has reducing properties in the unhydrolyzed state. A denatured lactalbumin preparation gave, prior to incubation, a reduction of only 6 per cent. After a 3 hour incubation with pepsin-hydrochloric acid a reduction of 41.4 per cent was noted. No further change was observed after 6 hours. The amount of iron entering into complex formation fell during digestion from 31.7 to 2.4 per cent. It appears therefore that digestion of insoluble proteins promotes reduction by putting protein in solution and increasing the amount of unbound iron.

The nature of the reducing groups, whether sulfhydryl or not, remains unsettled. Whatever it may be, they appear to act slowly, since lactalbumin digested for 3 hours gives little immediate reduction of added iron (about 14.6 per cent) but on 3 hours further incubation gave 39 per cent reduction, or a value very similar to that of the preceding experiments in which the iron was added at the beginning.

Further study of these protein effects, as well as of the influence of other reducing substances in foods, is required.

The methods used here also have been found applicable to experiments on reduction in the human stomach.

#### SUMMARY

A method is described for the estimation of ferric and ferrous forms of iron in the presence of each other. The method has been successfully applied to the determination of the degree of reduction and of complex formations by various foods and biological materials with ferric iron under conditions of artificial gastric digestion.

Fresh vegetables and fruits reduced the iron as much as 77 to 98 per cent. Ascorbic acid appears largely responsible. Egg white, meat, and bread reduced the iron to the extent of about 25 to 40 per cent. Milk gave less reduction and egg yolk none at all. Sugars produced little effect but proteins and their digestion products play a part in reduction of iron by certain foods. Further study is needed to determine more completely the nature of the substances involved in the reduction of the iron.

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## FREE AMINO ACIDS IN CEREBROSPINAL FLUID\*

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Values for total amino acid nitrogen and for certain individual amino acids in cerebrospinal fluid have been reported in the literature. The values for total  $\alpha$ -amino acid nitrogen show some variations, perhaps depending on the methods employed. Weichmann and Dominicke (1), using the Folin colorimetric method, report values of 1.2 to 2.0 mg. per cent, while Christensen *et al.* (2), using the manometric ninhydrin procedure, report 1.04 to 1.43 mg. per cent of amino nitrogen. Harris (3) has reported the glutamine content of cerebrospinal fluid to be 6.0 to 11.9 mg. per cent. Christensen *et al.* (2), using chemical methods, report the glycine values for spinal fluid to be about one-tenth and the alanine values to be about one-third of the corresponding values for blood plasma.

We are presenting results of the determination by microbiological methods of eleven individual amino acids in spinal fluid.

### EXPERIMENTAL

The microbiological determination of amino acids in spinal fluid offered some difficulty because of the small amounts of the amino acids present and because of the occurrence in spinal fluid of substances interfering with growth of the test organisms. An attempt to run estimations on spinal fluid without protein removal was unsuccessful because of growth inhibition. The use of tungstic acid as an agent for protein removal was not satisfactory. One difficulty lay in removing the protein completely without leaving an excess of tungstic acid in the filtrate.

We next tried heating spinal fluid at pH 4.7 to coagulate the protein and then filtering. Removal of protein was fairly complete but inhibition remained. We then evaporated this filtrate for 3 hours on a boiling water bath and found that the resulting solution could be successfully analyzed. The nature of the inhibiting substances destroyed by this treatment is not known. Our final procedure gave satisfactory recoveries of amino acids added to spinal fluid in amounts similar to those already present in the fluids. There were no significant changes in the  $\alpha$ -amino nitrogen values of

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spinal fluid treated according to our procedure, indicating that there was no hydrolytic liberation of amino acids involved.

### *Procedure*

The spinal fluid specimens analyzed were obtained from patients undergoing encephalography at the Illinois Neuropsychiatric Institute. Some of the patients were epileptic. All represented conditions in which no abnormality of the spinal fluid has been reported. There were no cases of syphilis, meningitis, or other inflammatory conditions. All specimens were clear and free from blood. The volumes varied usually from 50 to 100 cc., with some smaller specimens from children. The spinal fluids, collected by lumbar puncture under sterile conditions, were kept in a frozen state until analyzed. From 40 to 80 cc. of the spinal fluid were transferred to a 150 cc. beaker. The pH was adjusted with hydrochloric acid to 4.7 and the fluid then heated for 30 minutes on a boiling water bath. Coagulated protein was removed by filtration, the precipitate washed twice with 4 to 5 cc. of distilled water, and the filtrate returned to a beaker on the boiling water bath for about 2 hours longer. The fluids were thus concentrated to about one-fourth the original volume. The pH was adjusted to 6.8 with 0.1 N sodium hydroxide and diluted to a definite volume (one-fourth of the original volume).

The concentrated fluid was analyzed microbiologically for amino acids according to the procedures previously outlined for blood plasma and sweat (4, 5).

### RESULTS AND DISCUSSION

Table I gives the analyses of fluids from sixteen non-epileptic subjects. It will be seen that there are no great variations in the amount of the individual amino acids in the fluids from the various subjects.

Table II presents values for fluids from patients with epilepsy. It will be seen that the results for the individual amino acids fall in about the same range as the values in Table I (non-epileptic group). Means and standard deviations are given for the entire twenty-six spinal fluids. In Table II are also included values obtained on two composite samples of spinal fluid, one from adults (Group A (5), ages 45 to 62 years) and the other from children (Group B (5), ages 2 to 12 years). It will be seen that the values for the older age group are similar to those in Tables I and II, which included chiefly individuals from 25 to 60 years of age. The lower age group shows generally lower values for the individual amino acids. Further analyses in this lower age group are desirable. Children have been reported to show lower values than adults for blood-amino acid nitrogen (6).

In Table III a comparison is made of the levels of free amino acids in

spinal fluid with those in human plasma (4). The ratios of plasma values for spinal fluid are much lower, ranging from one-fifteenth to one-fourth of the values for plasma, with an average of about one-tenth. Arginine, with a ratio of 4, is somewhat exceptional. This somewhat independent position

TABLE I  
*Free Amino Acid Content of Cerebrospinal Fluid from Sixteen Non-Epileptic Subjects*

Reported as micrograms of amino acid per cc. of cerebrospinal fluid.

Specimen No.	Diagnosis	Age	Arginine	Histidine	Isoleucine	Leucine	Lysine	Phenylalanine	Threonine	Tyrosine	Valine	Methionine	Cystine
		yrs.											
1	Cortical atrophy	57 ♂	4.8	1.8	0.45	1.0	1.5	1.6	3.6	1.9	1.9		
2	Porencephaly	25 ♂	3.9	1.8	1.1	1.4	0.66	0.82	1.8	0.55	2.5		
3	Encephalopathy	27 ♂	6.4	1.5	1.6	1.0	2.4	1.8	1.8	2.1	1.9		
4	Tuberous sclerosis	26 ♂	7.7	2.0	2.7	1.3	2.0	2.0	3.1	1.6	2.6		
5	Encephalopathy	60 ♂	5.8	1.7	1.5	1.8	2.9	2.6	3.2	2.7	2.8		
6	Hemiathectomy	36 ♂	6.8	2.0	1.0	1.2	2.6	1.7	2.5	1.9	2.5		
7	Encephalopathy	30 ♂	7.4	1.8	1.3	1.4	2.8	2.7	2.3	2.4	2.3		
8	Cortical atrophy	34 ♀	3.5	1.4	0.78	0.95	3.5	1.6	2.3	1.9	1.6		1.6
9	"	42 ♀	3.7	1.6	0.71	1.4	3.2	1.7	1.9	2.0	1.6		1.7
10	Chorea, chronic	26 ♂	5.5	2.0	0.60	1.7	4.0	2.4	2.9	2.2	2.7	0.40	1.6
11	Cerebral thrombosis	38 ♂	6.5	1.4	0.50	1.3	3.5	2.5	2.5	2.4	1.6	0.43	2.7
12	Encephalitis lethargica	38 ♂	6.3	1.8	0.65	1.5	3.6	1.9	3.8	2.3	2.2	0.45	1.4
13	Cortical atrophy	28 ♂	5.7	1.4	0.65	1.6	3.4	1.7	3.7	1.6	2.2	0.28	1.6
14	Cerebral degenerative disease	52 ♀	6.7	1.5	0.60	1.4	3.0	2.3	2.7	2.0	2.1	0.38	2.7
15	Dilatation of cavum	25 ♀	6.0	1.9	0.75	1.7	3.9	1.4	3.9	1.9	2.5	0.43	0.85
16	Convulsive state	44 ♂	4.8	1.4	1.9	1.3	2.9		2.9	1.9	1.9		
Mean.....			5.7	1.7	1.04	1.3	2.8	1.9	2.8	1.9	2.1	0.39	1.8
S.D.....			1.3	0.2	0.5	0.2	1.0	0.5	0.6	0.6	0.7	0.08	0.4

of arginine is in agreement with results on sweat (5) which contains relatively much more arginine than blood plasma (4). The general patterns of the free amino acids in spinal fluid and plasma, while showing no extreme variations, are not closely similar. The mechanism by which such differential concentrations are maintained is not clear. It is believed, however, that these values for amino acids in spinal fluid, together with concentrations found after feeding large amounts of individual amino acids, will throw some further light on the physiology of the secretion.

We have also carried out some analyses on the protein of spinal fluid. The material used was a mixture of coagulated protein obtained from the spinal fluids assayed for amino acids. The protein so obtained was hydrolyzed by the method of McMahan and Snell (7). The pattern of free

TABLE II  
*Free Amino Acid Content of Cerebrospinal Fluid from Ten Epileptic Subjects*  
Reported as micrograms of amino acid per cc. of cerebrospinal fluid.

Specimen No.	Age	Arginine	Histidine	Isoleucine	Leucine	Lysine	Phenylalanine	Threonine	Tyrosine	Valine	Methionine	Cystine
	yrs.											
1	31 ♂	6.1	2.3	0.50	1.4	1.9	1.6	2.4	1.9	2.1		
2	28 ♀	6.6	2.5	0.45	1.5	3.0	1.3	2.2	2.9	2.1		
3	26 ♀	6.4	1.4	1.0	1.7	2.8	1.1	1.4	1.6	1.1		
4	36 ♂	6.3	2.0	2.2	1.2	2.0	2.4	2.3	1.9	1.9		
5	34 ♂	6.9	1.9	0.94	1.0	1.7	2.1	3.5	2.2	1.5		
6	29 ♀	6.8	2.1	1.1	1.6	2.1	2.5	3.6	2.5	2.8		
7	26 ♂	5.5	2.0	0.70	1.4	2.9	2.6	3.2	2.6	1.8	0.29	2.5
8	45 ♂	6.7	1.6	0.60	1.8	4.4	3.1	3.0	3.2	2.7	0.47	2.0
9	57 ♂	8.2	1.4	0.55	1.3	3.1	2.0	3.9	1.8	2.1	0.45	2.4
10	27 ♂	6.6	1.5	0.75	1.5	2.9	1.5	3.8	2.0	2.5	0.47	1.5
Mean.....		6.6	1.8	0.87	1.4	2.6	2.0	2.9	2.2	2.0	0.42	2.1
S.D.....		0.7	0.3	0.4	0.2	0.7	0.6	0.7	0.5	0.5	0.07	0.4

Recapitulation for all subjects

Combined mean*..	6.0	1.7	0.98	1.4	2.8	1.9	2.8	2.0	2.1	0.40	1.8
S.D.....	1.4	0.5	0.5	0.2	0.8	0.7	0.9	0.7	0.5	0.09	0.5

Comparison of two composite samples

Group A, old.....	5.0	1.7	0.68	1.2	2.4	1.8	2.7	2.4	1.6	0.36	1.2
" B, young.....	2.6	0.70	0.41	0.81	1.4	0.80	1.3	1.1	1.1	0.20	0.60

\* Twenty-six specimens.

amino acids in blood plasma has been shown to follow quite closely the pattern of the combined amino acids in the blood plasma proteins, suggesting some relationship. The free amino acids of spinal fluid do not follow closely the pattern of the amino acids in the spinal fluid protein, which suggests a lack of relationship. In spinal fluid (Table III) the ratio of the concentration of arginine to that of histidine is 3.5, while in the protein of spinal fluid

the ratio is 1.0. The ratio of the tyrosine value to the valine value in spinal fluid is 0.9, while in the spinal fluid protein the ratio is approximately 0.4.

The values for spinal fluid protein show a certain similarity with those for ox serum albumin (8) and would be reasonably consistent with the view that the spinal fluid protein is a combination of serum albumin with some pseudoglobulin and perhaps small amounts of other proteins. It is generally believed that the protein of spinal fluid consists largely of serum albumin. Dr. C. A. Johnson tested some of our specimens, using the precipitin reaction, for human serum albumin and confirmed this. He also showed, by use of the precipitin reaction, that small amounts of human pseudoglobulin were present.

TABLE III

*Comparison of Free Amino Acids in Cerebrospinal Fluid with Those in Human Plasma and in Amino Acids of Cerebrospinal Fluid Protein*

Amino acids	Free amino acids,* mean and s.d.		Ratio, Plasma Spinal fluid	Spinal fluid protein†
	Spinal fluid	Plasma		
Arginine.....	6.0 ± 1.4	23 ± 6	4	5.42
Histidine.....	1.7 ± 0.5	14 ± 2	8	5.16
Isoleucine.....	0.98 ± 0.5	16 ± 3	15	1.75
Leucine.....	1.4 ± 0.2	20 ± 3	15	6.22
Lysine.....	2.8 ± 0.8	29 ± 4	10	13.05
Phenylalanine.....	1.9 ± 0.7	14 ± 3	8	3.16
Threonine.....	2.8 ± 0.9	20 ± 4	7	4.06
Tyrosine.....	2.0 ± 0.7	15 ± 4	8	1.97
Valine.....	2.1 ± 0.5	28 ± 3	14	5.00

\* Reported as micrograms of amino acid per cc. of cerebrospinal fluid and plasma.

† Reported as amino acid nitrogen in per cent of total nitrogen.

The possibility of the presence in spinal fluid of amino acids as "conjugates" or in peptide combination is recognized. The presence of such forms in plasma has been reported (9). The values of Harris (3) for glutamine in cerebrospinal fluid comprise about 69 per cent of the spinal fluid total  $\alpha$ -amino nitrogen. Christensen *et al.* (2) report the glycine concentration of spinal fluid as being one-tenth and the alanine concentration one-third of the plasma levels. Gutman and Alexander (10) report plasma levels of 8.0 and 16.1 mg. per cent for glycine and alanine respectively. Our results on eleven individual amino acids show them to constitute approximately 18 per cent of the total  $\alpha$ -amino nitrogen reported for cerebrospinal fluid (2). On adding the values for glutamine, glycine, alanine, and the values for the eleven individual amino acids, one gets a value of approximately 94 per cent of the total, using the average value (8.95 mg. per cent) for glutamine as

reported by Harris (3). We were not able to demonstrate the presence of free proline in spinal fluid. Our results with glutamic acid indicate that it is present in amounts not greater than 2 per cent of the glutamine value. On the basis of these findings it appears improbable that the presence of "conjugates" or other peptide combinations has a great effect, if any, on the determination of the individual amino acids in spinal fluid by the microbiological methods employed.

#### SUMMARY

A method for the microbiological determination of free amino acids in cerebrospinal fluid is described. Results are presented for eleven individual free amino acids in the spinal fluid of twenty-six subjects. Average values, in micrograms per cc. were as follows: arginine  $6.0 \pm 1.4$ , histidine  $1.7 \pm 0.5$ , isoleucine  $0.98 \pm 0.5$ , leucine  $1.4 \pm 0.2$ , lysine  $2.8 \pm 0.8$ , phenylalanine  $1.9 \pm 0.7$ , threonine  $2.8 \pm 0.9$ , tyrosine  $2.0 \pm 0.7$ , valine  $2.1 \pm 0.5$ , methionine  $0.4 \pm 0.09$ , and cystine  $1.8 \pm 0.5$ . The values vary from about one-fourth to one-fifteenth of those for blood plasma.

No significant changes were noted in the free amino acids in the spinal fluids of epileptic or other patients studied.

An analysis of spinal fluid protein is reported.

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## DIPHTHERIA TOXIN

### IV. THE IRON ENZYMES OF *CORYNEBACTERIUM DIPHTHERIAE* AND THEIR POSSIBLE RELATION TO DIPHTHERIA TOXIN\*

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In the previous communication (1) the inhibitory effect of iron on toxin and porphyrin production by the Toronto strain of *Corynebacterium diphtheriae* was reported. It was shown that for every 4 atoms of iron added to the culture medium (over and above that optimal for toxin production) 4 molecules of porphyrin and 1 of toxin failed to appear in the culture supernatant. All of the iron added to the medium over the range of toxin inhibition could be recovered from the bacterial cells, and as their iron content increased a two banded spectrum became visible in cell suspensions treated with dilute alkali and sodium hydrosulfite. On the basis of these observations it was suggested that diphtheria toxin may be the protein moiety of an iron-containing respiratory enzyme.

In the present study, we have investigated the effect of iron on production of the following enzymes by diphtheria bacilli: catalase, cytochromes *b* and *c*, and cytochrome oxidase. While proof is lacking, the results furnish evidence that diphtherial cytochrome *b* may be the enzyme postulated to contain iron, porphyrin, and toxin.

#### Methods

**Cultural Methods**—The Park-Williams No. 8 (Toronto) strain of *Corynebacterium diphtheriae* was grown on Mueller and Miller's medium (2) in the manner described in the previous communication (1). Organisms were harvested after 5, 6, and sometimes 7 days growth at 34°. For metabolic studies, it has been found that the final pH at which the organisms are harvested is of greater importance than the time of incubation. Bacterial suspensions from cultures harvested between pH 6.5 and 7.5 show reproducible and maximal enzymic activity. Reproducible results are not obtained when the final pH is above 7.5, and such bacterial suspensions often show greatly diminished catalase and oxidase activity.

In addition to the Toronto strain, which is of the *intermedius* type, some

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experiments have also been carried out with the Halifax strain of *Corynebacterium diphtheriae*. This strain is a typical *gravis* type isolated in the Halifax epidemic in 1940 and studied by Mueller (3). The Halifax strain produces relatively little toxin at low iron concentrations (<10 Lf per cc. as compared with 70 Lf per cc. by the Toronto strain under identical conditions). Toxin production by the Halifax strain is not inhibited to quite the same extent at high iron concentrations as is the case with the Toronto culture (3).

**Bacterial Suspensions**—Bacteria from 300 cc. of culture were collected by centrifugation and washed three times with 250 cc. charges of saline. The washed organisms were then made up to 20 to 25 cc. and shaken for 1 minute in a 9000 cycle sonic oscillator.<sup>1</sup> Treatment for this short period yielded homogeneous suspensions with only slight disruption of the cells. The suspensions were diluted 200-fold and the turbidity read in a Coleman spectrophotometer at 650 m $\mu$ . Bacterial nitrogen per cc. was estimated from a standard curve.

**Determination of Catalase Activity**—Catalase activity of whole washed organisms was determined by measuring the rate of decomposition of hydrogen peroxide in 0.0067 M phosphate buffer at pH 6.8 and 0° according to the method outlined by Sumner and Somers (4). The iodine liberated from potassium iodide by hydrogen peroxide after different time intervals was titrated with sodium thiosulfate. Catalase units (*Kat. f.*) are expressed as the monomolecular constant at zero time per gm. of bacteria.

**Respiration Experiments**—Oxygen consumption was measured in the Warburg apparatus at 36.5° in the usual manner. Unless otherwise noted, 0.25 cc. of 0.16 M substrate was placed in the side arm, 0.2 cc. of 20 per cent NaOH in the center cup, and 0.5 cc. of 0.4 M phosphate buffer at pH 7.3 in the vessel itself. The total volume was 2 cc. in all cases. Whenever whole organisms were used as a source of enzyme, blank runs were carried out without substrate.  $Q_{O_2}$  values are expressed as c.mm. of O<sub>2</sub> per mg. of bacterial nitrogen per hour, calculated from the oxygen consumed during the first 30 minutes, and are corrected for "resting" metabolism.

#### EXPERIMENTAL

**Effect of Iron on Catalase Production**—The effect of adding varying amounts of iron to the culture medium on catalase production by the Toronto and Halifax strains is shown in Table I. The cultures to which no iron was added had been adjusted previously to the iron concentration optimal for toxin production by the Toronto strain. It will be noted that with the Toronto strain, catalase production is proportional to the iron added, and therefore varies inversely with toxin production. As shown

<sup>1</sup> Manufactured by the Submarine Signal Corporation of Boston, Massachusetts.

previously (1), the iron content of cells grown in the presence of an excess of iron is about 5 times that of organisms grown under conditions most favorable for toxin production. The increase in catalase content is also approximately 5-fold. Crystalline catalase contains 30,000 to 60,000 units per gm. and its iron content is 0.09 per cent (4). From these figures and the data given in Table I it may be calculated that the increase in catalase iron over the range in question is only about 10  $\gamma$  per gm. of bacteria. Since the total increase in bacterial iron content is 70 to 80  $\gamma$  per gm., it seems most unlikely that catalase can be the postulated toxin-containing iron enzyme.

It will also be noted from Table I that, even in the presence of excess iron, only 10 per cent as much catalase is produced by the Halifax strain as by the Toronto strain. Moreover, the catalase content of the former strain is only increased 65 per cent by the addition of 200  $\gamma$  of iron per 300 cc. of culture medium as compared with an increase of almost 500 per cent

TABLE I  
*Effect of Iron on Catalase Production*

Iron added	Park-Williams No. 8 (Toronto) strain, <i>intermedius</i>		Halifax strain, <i>gravis</i>	
	Growth	Catalase	Growth	Catalase
$\gamma$ per 300 cc.	mg. N	Kat. f.	mg. N	Kat. f.
0	162	185	54	52
50	215	400	153	76
100	228	610	163	78
200	240	840	186	86

for the Toronto strain. This may be attributed to the failure of the Halifax strain to grow satisfactorily at low iron concentrations, which support heavy growth of the Toronto culture. While at high iron levels the Halifax strain yields 75 to 80 per cent as much bacterial nitrogen as the Toronto strain, only about 30 per cent as much growth is obtained when the iron concentration is reduced to that optimal for toxin production by the Toronto culture. Presumably, the change in cellular iron content of organisms of the Halifax strain is less than 2-fold. Whether the failure of this strain to yield heavy growth at low iron concentrations is due to its low catalase content or to some other reason has not been determined.

*Effect of Iron on Succinate Oxidation*—Measurement of the rate of oxidation of succinate by cell suspensions may often give an approximate estimate of the over-all activity of the cytochrome system. The effect of adding iron to the culture medium on the rate of oxygen uptake by washed suspensions of diphtheria bacilli is shown in Table II. The results are



similar to those obtained with catalase. Oxygen uptake by suspensions of the Toronto strain is proportional to the iron added and varies inversely with toxin production. Suspensions of high iron content oxidize succinate 5 to 6 times faster than organisms grown under conditions favorable for maximal toxin production. As is seen from Table II, the Halifax strain is somewhat more active than the Toronto strain in the presence of excess iron. However, the  $Q_{O_2}$ -succinate for the Halifax strain is increased only 60 to 70 per cent over the iron range in question. As in the case of catalase, this may be attributed to the relatively high iron content of organisms of the Halifax strain grown on media deficient in iron.

Similarly with other substrates, including glucose, maltose, lactate, fumarate, malate, glutamate, and  $\alpha$ -ketoglutarate, an increased rate of oxidation was found with suspensions of Toronto organisms of increasing iron content.

TABLE II  
*Effect of Iron on Succinate Oxidation*

Iron added	Park-Williams No. 8 (Toronto) strain, <i>intermedius</i>		Halifax strain, <i>gravis</i>
	Toxin	$Q_{O_2}$	$Q_{O_2}$
$\gamma$ per 300 cc.	Lf per cc.	c.mm. $O_2$ per mg. N per hr.	c.mm. $O_2$ per mg. N per hr.
0	70	20-30	81
20	62	35-50	
50	48	43-66	82
70	40	72	
100	25	110	129
200	0	106-120	137

*Spectroscopic Examination of Bacterial Extracts*—When heavy suspensions of diphtheria bacilli containing 5 to 10 mg. of bacterial nitrogen per cc. are treated for 30 minutes in the 9000 cycle sonic oscillator, the cells are almost completely disrupted. Smears from the suspensions following this treatment show only occasional intact organisms. After removing the cellular debris in a Swedish angle centrifuge, opalescent extracts may be obtained which, when fresh, show 90 per cent of the activity of the original cell suspension when tested for oxygen uptake in the presence of succinate.

Such extracts from cells of high iron content are deep reddish brown in color. On adding a little sodium hydrosulfite, the color changes to a more pinkish hue and a two banded hemochromogen type of spectrum becomes clearly visible when viewed with a small spectroscope. On the other hand, extracts from organisms of low iron content grown under conditions optimal for toxin production are pale straw color and show no distinct bands in

the spectroscope. The absorption spectra in the visible region of *reduced* diphtherial extracts of high and low iron content in phosphate buffer at pH 7.2 are shown in Fig. 1. The measurements were made in the Beckman spectrophotometer with sonic extracts which had been partially clarified by centrifugation in the refrigerated centrifuge at 18,000 R.P.M. for 30 minutes. Despite the relatively large general absorption of the crude

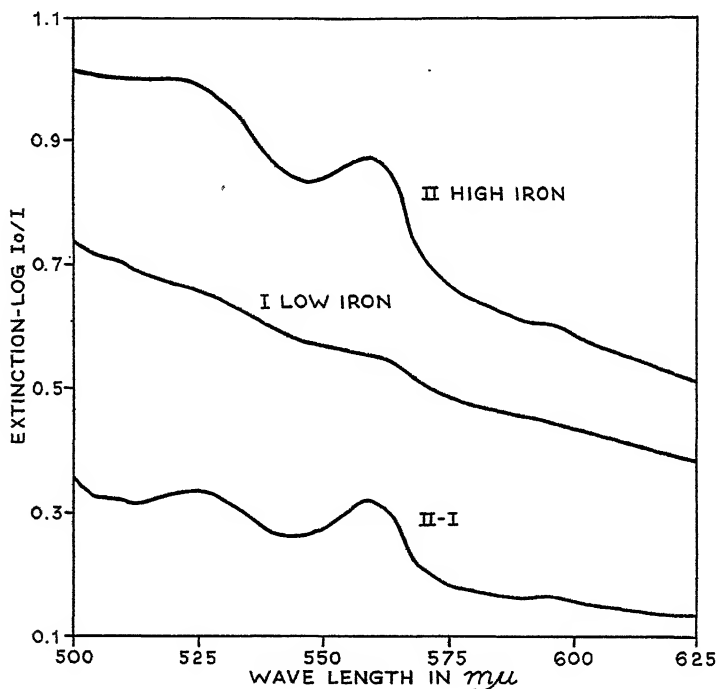


FIG. 1. Absorption spectrum of reduced cytochrome *b* in extracts of *Corynebacterium diphtheriae* (visible region). Curve I, extract from organisms of low Fe content; 6.0 mg. of bacterial nitrogen per cc. in 0.13 M phosphate buffer at pH 7.2 clarified at 18,000 R.P.M. for 30 minutes. Curve II, extract from organisms of high Fe content; 5.3 mg. of bacterial nitrogen per cc. in 0.13 M phosphate buffer at pH 7.2 clarified at 18,000 R.P.M. for 30 minutes.

extracts, pronounced maxima at 560  $m\mu$  and 524 to 525  $m\mu$  are clearly visible. In Fig. 2 are plotted the absorption spectra of diluted extracts from cells of high iron content in the near ultraviolet before and after reduction with hydrosulfite. The curves shown in Fig. 2 were obtained by subtracting the absorption of extracts of low iron content from that of high iron-containing extracts. The intense Soret band at 415  $m\mu$  is shifted to 429  $m\mu$  following reduction.

Fujita and Kodama (5) in their spectroscopic study of bacterial cytochromes reported that cytochrome *b* was the major cytochrome component observed in suspensions of diphtheria bacilli. Our own findings are in

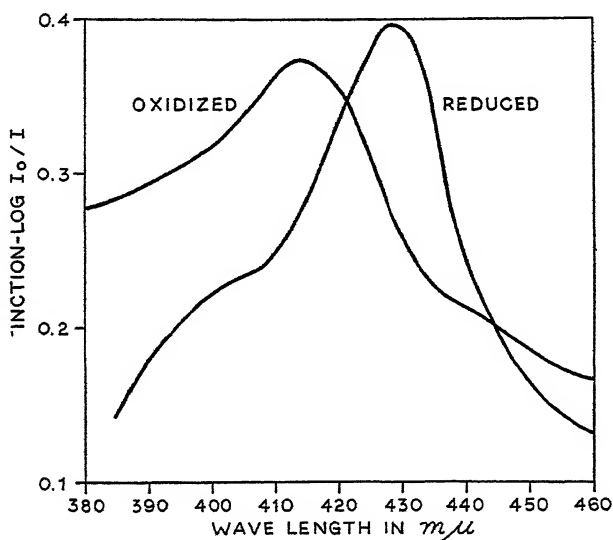


FIG. 2. Absorption spectrum of cytochrome *b* in extracts of *Corynebacterium diphtheriae* (near ultraviolet). Extract from organisms of high Fe content read against extract from organisms of low Fe content before and after reduction with sodium hydrosulfite. 1 mg. of bacterial nitrogen per cc. in 0.13 M phosphate buffer at pH 7.2 clarified at 18,000 R.P.M. for 30 minutes.

TABLE III  
*Absorption Maxima of Reduced Cytochrome Components*

Component	Source	Bibliographic reference	Absorption maxima		
			mμ	mμ	mμ
<i>a</i>	Heart muscle	Keilin and Hartree (6)	605	?	452
<i>a</i> <sub>2</sub>	" "	" " " (6)	600	?	448
<i>b</i>	" "	" " " (6)	564	530	432
"	Diphtheria bacillus	Fujita and Kodama (5)	562	532	?
"	" "	Present study	560	524	429
<i>b</i> <sub>2</sub>	Yeast	Bach, Dixon, and Zervas (7)	556.3	530	?
<i>c</i>	Heart muscle	Keilin and Hartree (6)	550	521	415

agreement with their observations. The position of the bands of diphtherial cytochrome *b* in relation to other cytochrome components from various sources is summarized in Table III. The spectrum of diphtherial cytochrome *b* is unaltered in the presence of 0.003 M potassium cyanide.

Fujita and Kodama (5) reported a weak band at 550 m $\mu$ , corresponding to cytochrome *c*, in suspensions of diphtheria bacilli. In our extracts we have been unable to identify with certainty bands due to any of the cytochrome components other than cytochrome *b*. An extract from 3 gm. of diphtheria bacilli of high iron content was fractionated and concentrated to a volume of 2.5 cc. by the method of Stotz (8) for quantitative estimation of cytochrome *c* in tissues. No increase in rate of oxygen uptake occurred when this concentrate was added to phenylenediamine and a cytochrome oxidase preparation from rabbit kidney cortex. On the other hand, the rate of oxygen uptake by the test system was increased 50 per cent upon addition of 0.2 mg. of cytochrome *c*.<sup>2</sup> Thus if cytochrome *c* is present in the bacterial extracts, it is as a minor component.

*Effect of Cyanide on Succinate Oxidation by Whole Organisms and by Cytochrome b-Containing Extracts*—Fujita and Kodama (5) noted that the respiration of suspensions of diphtheria bacilli was only 20 per cent inhibited by cyanide. With fresh suspensions of whole organisms harvested between pH 6.5 and 7.5, we have observed as high as 30 to 40 per cent inhibition of succinate, glucose, and lactate oxidation by  $2.5 \times 10^{-3}$  M KCN. If the pH of the culture medium rises above 7.5 during growth, bacterial suspensions oxidize succinate somewhat less rapidly and are less sensitive to cyanide. Bacterial suspensions which have been stored in the cold for a few days also lose that fraction of succinoxidase activity which is inhibited by cyanide. As we have already pointed out, extracts from bacteria disrupted by sonic vibration show negligible loss in activity when tested immediately for oxygen uptake in the presence of succinate. After 30 to 40 minutes centrifugation at 18,000 R.P.M. to remove the larger cell fragments, such extracts are still inhibited 10 to 20 per cent by KCN. As with whole organisms, a slight loss in activity occurs on standing and the preparation becomes completely insensitive to cyanide inhibition within a few days. Extracts which have become cyanide-insensitive are very stable and retain their activity for weeks when stored in the cold in solution containing 1:10,000 merthiolate. The effect of cyanide on oxygen uptake by organisms of the Toronto strain and fresh extracts from them is shown in Table IV. The effect of cyanide on the oxidation of phenylenediamine by cell-free extracts is also shown in Table IV.

Fumarate and malate are oxidized by fresh bacterial suspensions at a slower rate than succinate and the oxidation is almost completely (>80 per cent) inhibited by  $2.5 \times 10^{-3}$  M KCN. If succinate is not present in excess, oxygen uptake proceeds at a progressively slower rate until approximately 0.5 mole of oxygen has been consumed per mole of substrate. The oxidation then proceeds at a constant rate characteristic of fumarate or malate,

<sup>2</sup> Prepared from beef heart by the method of Keilin and Hartree (9).

as shown in Fig. 3. No oxidation of fumarate or malate takes place in the absence of intact cells.

*Properties of Diphtherial Cytochrome b*—The cytochrome *b* in extracts from the diphtheria bacillus is readily autoxidizable and its autoxidation is unaffected by the presence of 0.003 M KCN. When succinate is added to diphtherial extracts in a Thunberg tube under anaerobic conditions, the bands of reduced cytochrome *b* quickly become visible in a small spectro-scope. This reduction has been followed at 560 m $\mu$  by means of small Thunberg tubes designed to fit the Beckman spectrophotometer. When a final concentration of 0.025 M sodium succinate is added to active ex-

TABLE IV  
*Effect of KCN on O<sub>2</sub> Uptake by Toronto Strain Grown in Excess Iron*

Preparation	Substrate	KCN	Q <sub>O<sub>2</sub></sub> *	Inhibi- tion
		moles per l.	c.mm. O <sub>2</sub> per mg. N per hr.	per cent
Whole organisms	Succinate	0	85	0
		$2.5 \times 10^{-4}$	60.5	29
		$2.5 \times 10^{-3}$	54.5	36
Sonic extract clarified 45 min. at 18,000 R.P.M.	"	0	75	0
		$2.5 \times 10^{-5}$	66	12
		$2.5 \times 10^{-4}$	59	20
Same	Phenylenediamine	$2.5 \times 10^{-3}$	59.5	19.5
		0	17	0
		$2.5 \times 10^{-4}$	1	94
Extract from organisms of low Fe content clarified at 18,000 R.P.M.	"	$2.5 \times 10^{-3}$	0	100
		0	4.1	0

\*Q<sub>O<sub>2</sub></sub> values in this case are expressed per mg. of bacterial N of the original suspension of intact organisms.

tracts containing 2 mg. of nitrogen per cc. in phosphate buffer at pH 7.2 (Q<sub>O<sub>2</sub></sub> = 160 c.mm. of O<sub>2</sub> per mg. of N per hour), 50 to 60 per cent reduction of cytochrome *b* occurs within a few minutes. When air is admitted to the tube, the intensity of the absorption at 560 m $\mu$  diminishes, indicating its autoxidation. Identical results are obtained when the same experiment is carried out in the presence of 0.003 M KCN. These experiments furnish strong evidence that cytochrome *b* is directly concerned in the oxidation of succinate by the diphtheria bacillus. It will be recalled that the oxidation potential of the succinate-fumarate system lies some 40 millivolts above that of cytochrome *b* at pH 7 (*E*<sub>0</sub> = 0.00 and -0.04 volts respec-

tively; Ball (10)). It is not surprising, therefore, that reduction of cytochrome *b* by succinate is not complete. In this connection it is of interest that oxidation of succinate by cytochrome *b*-containing extracts always proceeds at a much faster rate during the first few minutes after adding the substrate to the enzyme preparation. The rate then slows down and becomes relatively constant. Presumably, as fumarate is formed, the

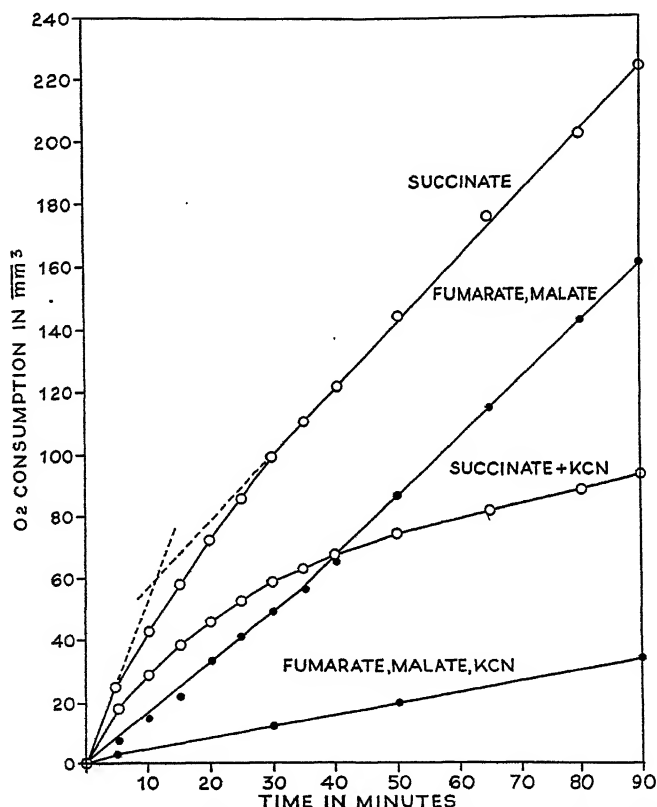


FIG. 3. Effect of KCN on oxygen consumption by diphtheria bacilli of high Fe content. Total volume per vessel, 2.0 cc.; sodium succinate  $\cdot 6\text{H}_2\text{O}$ , 2.0 mg.; fumarate or malate, 0.025 M; phosphate buffer pH 7.3, 0.1 M; KCN, 0.0025 M; temperature  $36.5^\circ$ .

oxidation potential of the substrate is raised, thereby reducing the efficiency of the catalyst.

At least a portion of the cytochrome *b* in sonic extracts of diphtheria bacilli appears to be bound to large molecular components. If extracts which have been clarified by centrifugation at 18,000 R.P.M. for 30 minutes are sedimented for an additional 2 hours at this speed in the refrigerated

ultracentrifuge, about 70 per cent of the cytochrome *b* accumulates as a red button at the bottom of the tube. The sedimented material also possesses about 70 per cent of the succinoxidase activity, indicating that no separation of succindehydrogenase from cytochrome *b* is achieved by this differential centrifugation. The  $Q_{O_2}$  of the sedimented succinoxidase is approximately twice that remaining in the clear supernatant. It seems not unlikely that a part of the cytochrome *b* is made soluble by sonic treatment, as were the cytochrome oxidase preparations of Haas (11) treated with ultrasonic vibration.

TABLE V

*Effect of Inhibitors on Succinate Oxidation by Cytochrome b-Containing Extracts*  
Succinate concentration 0.02 M in each case.

Inhibitor	Concentration	$Q_{O_2}$	Inhibition
	<i>moles per l.</i>	<i>c.mm. O<sub>2</sub> per mg. N per hr.</i>	<i>per cent</i>
None		152	
KCN	$2.5 \times 10^{-3}$	144	5
None		160	
Malonate	0.025	52.5	74
	0.1	7.7	95
None		160	
Arsenite	$10^{-3}$	156	2.5
	$10^{-2}$	136	15
None (pH 6.1)		51	
	<i>mg. per l.</i>		
Naphthoquinone	1	58	
	10	54.5	
	100	51	0
None (pH 7.2)		171	
Naphthoquinone	1	172	0
	10	171	0
	100	172	0

In Table V is summarized the effect of various inhibitors on the cell-free diphtherial succinoxidase system. As with mammalian succinoxidase preparations, malonate is a powerful inhibitor. Arsenite causes slight inhibition in 0.01 M concentration, while potassium cyanide and the hydroxynaphthoquinone, SN 5949,<sup>3</sup> are without effect on the rate of succinate oxidation. The failure of compound SN 5949 to inhibit succinate oxidation is of particular interest, since Ball and his associates (12) have recently shown that the heart muscle succinoxidase system is completely inhibited

<sup>3</sup> We are greatly indebted to Dr. Eric G. Ball of Harvard Medical School for kindly sending us a generous sample of compound SN 5949, 2-hydroxy-3-( $\beta$ -methyloctyl)-naphthoquinone.

by this substance at concentrations as low as  $3 \times 10^{-6}$  M and that the inhibitor acts at a point just below cytochrome *c* in the chain of respiratory enzymes. Its failure to inhibit the diphtherial succinoxidase system furnishes additional evidence that some intermediate enzyme linking cytochromes *b* and *c* is acted upon, as was suggested by Ball *et al.* (12).

*Effect of Iron on Cytochrome Oxidase Production*—Despite our failure to demonstrate cytochrome *c* in extracts from the diphtheria bacillus, it seems likely that this component is present in small amounts. At any rate, fresh cell-free extracts are capable of slowly oxidizing hydroquinone and phenylenediamine at a rate equivalent to that fraction of the succinoxidase activity which is inhibited by cyanide (see Table IV). The enzyme system concerned in phenylenediamine oxidation is completely inhibited by 0.0025 M KCN. Its production is increased with increasing iron concentration in the culture medium, as are catalase and cytochrome *b*. The system is labile and is associated to a large extent with the heavier cell fragments, as is mammalian cytochrome oxidase. Addition of cytochrome *c* to the preparation results in a slight but definite increase in  $Q_{O_2}$  (10 to 20 per cent). Neither phenylenediamine nor hydroquinone is oxidized by intact diphtheria bacilli, presumably because the cell is impermeable to these substrates.

#### DISCUSSION

In the previous paper (1) it was shown that diphtheria bacilli grown on media containing an excess of iron have a cellular iron content about 5 times greater than that of organisms grown under conditions most favorable for toxin production. Moreover, the amount of highly specific toxic protein which fails to be excreted by the cells is equivalent to the iron added. It may be expected, therefore, that the proposed toxin-containing iron enzyme will be the major respiratory pigment of cells of high iron content. The present studies have demonstrated that the major iron porphyrin-containing pigment of the diphtherial cell is spectroscopically related to cytochrome *b*, as indeed was first shown in 1934 by Fujita and Kodama (5). The extinction coefficient of pure cytochrome *b* is not known and it is therefore impossible at this time to demonstrate that the increase in cytochrome *b* content of the cells is equivalent to the iron taken up and the toxin which fails to appear in the supernatant. Nevertheless, comparison of the change in absorption by diphtherial extracts of increasing iron content at 560 m $\mu$ , following reduction with hydrosulfite, with the change in absorption of pure cytochrome *c* at 550 m $\mu$ , following reduction, suggests that the cytochrome *b* content of diphtheria bacilli is of an order of magnitude equivalent to their iron content. Our results thus lead us to the tentative conclusion that diphtherial cytochrome *b* is a pigment composed of iron, porphyrin, and diphtheria toxin.



If the above conclusion is indeed the correct one, it seems logical to assume that diphtheria toxin interferes in some manner with the functioning of cytochrome *b* or some closely related enzyme in the tissues of susceptible animals. A proper understanding of the rôle of cytochrome *b* in mammalian tissue respiration therefore becomes of considerable significance in this connection. The function of cytochrome *b* is still an unsettled question. Nevertheless, there is a good deal of evidence that cytochrome *b* is concerned in the oxidation of succinate by heart muscle preparations, in which it may serve as a link between succinate dehydrogenase and the cytochrome *c*-cytochrome oxidase system (Keilin and Hartree (6), Ball *et al.* (12), Stotz (13)). There is no evidence, at present, that mammalian tissue preparations containing cytochrome *b* can oxidize succinate in the absence of cytochrome *c* and cytochrome oxidase unless some other catalyst such as methylene blue is added. Nor has any separation of succinate dehydrogenase from cytochrome *b* been effected.

The succinoxidase system of the diphtheria bacillus differs in several important respects from that of heart muscle. Cytochrome *b* is the major cytochrome component present. The spectral bands of diphtherial cytochrome *b* lie 3 to 4  $m\mu$  to the left of those found in heart muscle, and the bacterial pigment appears to be more rapidly autoxidizable than that from animal sources. The most striking difference between the diphtherial succinoxidase system and that of heart muscle, however, is the relative insensitivity of the former system to inhibition by cyanide and the hydroxynaphthoquinone, SN 5949. Both of these inhibitors have been shown to act at points which lie above cytochrome *b* in the respiratory chain. The rate of oxidation of succinate by cyanide-insensitive extracts is roughly proportional to their cytochrome *b* content, as estimated from the change in intensity of their absorption at 560  $m\mu$  following reduction with sodium hydrosulfite. It seems probable, from the evidence presented, that cytochrome *b* is the limiting factor in succinate oxidation by cyanide-insensitive cell-free diphtherial extracts.

In contrast to succinate, the oxidation of fumarate and malate is more than 80 per cent inhibited by cyanide. This suggests that the respiratory pathway is different for these latter substrates and does not involve cytochrome *b*.

Cell-free extracts from sonically disrupted diphtheria bacilli of both high and low iron content yield only traces of precipitate when tested against a rabbit antitoxic precipitating serum. Thus cytochrome *b* is serologically different from diphtheria toxin. Moreover, diphtherial cytochrome *b* is not toxic, since only traces of toxin (less than 10 M.L.D. per cc.) are present in extracts from heavy suspensions of diphtheria bacilli of either high or low iron content. This is in agreement with the observations of Morton and Gonzalez (14) who found less than 0.1 per cent as much toxin

inside diphtherial cells as in the culture filtrate. If cytochrome *b* were specifically precipitated by rabbit antitoxin, direct evidence for its relationship to diphtheria toxin would be provided. Its failure to react with antitoxin, however, does not invalidate the hypothesis, for which we have already furnished considerable indirect evidence, that such a relationship does exist. Indeed it would be surprising if incorporation of four hemin groups into the toxin molecule would leave its immunological specificity unaltered. In addition, it should be recalled that cytochrome *b* is bound to the larger molecular components of the diphtherial cell, which might also bring about a change in specificity. However, until some specific effect of toxin on cytochrome *b* activity in the intoxicated animal can be demonstrated, or cytochrome *b* can be dissociated into hemin and a toxic component, or a substance possessing cytochrome *b* activity can be synthesized from hemin and diphtheria toxin, final proof of the relationship is wanting.

#### SUMMARY

1. The effect of iron on the production of catalase, cytochromes *b* and *c*, and cytochrome oxidase by two strains of *Corynebacterium diphtheriae* has been studied.

2. The major iron porphyrin-containing pigment of the diphtheria bacillus is cytochrome *b*. Evidence is presented which suggests a possible relationship between cytochrome *b* and diphtheria toxin.

3. The oxidation of succinate by cell-free extracts of the diphtheria bacillus has been studied. The bacterial suspension differs from the heart muscle succinoxidase system in its relative insensitivity to inhibition by cyanide and the naphthoquinone SN 5949.

4. Cytochrome *b* appears to be the limiting factor concerned in the oxidation of succinate by the cell-free bacterial extracts.

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## STUDIES ON CHOLINESTERASE

### IV. ON THE MECHANISM OF DIISOPROPYL FLUOROPHOSPHATE ACTION IN VIVO\*

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There is growing evidence for the assumption that the profound biological effects produced by some compounds in low concentrations may be attributed to an interaction with enzymes. This is true for certain cell constituents as well as for toxic compounds not existing normally in the body. Several vitamins have been found to be prosthetic groups of enzymes and for a number of drugs it could be demonstrated that the effect has to be attributed to their action on either the protein or non-protein moiety of an enzyme system. When it became necessary to study certain toxic compounds considered as potential agents in chemical warfare, English investigators, as stressed by Peters and his associates (1) and Dixon and Needham (2), approached the problem based on the assumption that a probable explanation for the mode of action may be the effect of these compounds on enzymes.

According to Dixon and Needham, the most powerful and most specific enzyme inhibitor known is the diisopropyl fluorophosphate (DFP). This compound may inhibit cholinesterase in concentrations as low as  $1 \times 10^{-10}$  M. Although twenty enzyme systems were studied, none of them was affected except the esterases. The toxic symptoms in animals exposed to DFP indicate that the central nervous system is affected. It is known that all nervous tissue contains cholinesterase in high concentrations (3, 4). Much evidence has accumulated in support of the paramount importance of this enzyme for nerve conduction (5-8). The discovery of DFP offered an unusual tool to test this assumption. This compound inactivates cholinesterase irreversibly. This irreversible inactivation is, however, not an immediate process, as was first believed, but requires a certain period of time (9, 10). Owing to this peculiar feature of this compound, a striking parallelism has been established in nerves exposed to DFP between the progressive irreversible inactivation of cholinesterase and the abolition of conduction. In view of all these facts and since abolition of conduction is

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incompatible with life, it appeared reasonable to assume that the high toxicity of DFP *in vivo* has to be attributed to its effect on cholinesterase.

Mazur and Bodansky (11) showed, however, that animals treated with DFP could survive in the presence of insignificant small concentrations and even in complete absence of cholinesterase in brain. This seemed then to indicate that the toxicity of the compound is not directly related to the effect on cholinesterase. The authors considered, indeed, the small amounts found in some surviving animals as fortuitous and not necessary for survival.

In view of the fundamental questions and principles involved, and of the fact that all observations on isolated nerves exposed to DFP are in contradiction to the conclusion mentioned, the problem has been reexamined and the results obtained are reported in this paper.

### *Methods*

The activity of cholinesterase was determined with the manometric technique as applied previously (12). The different ways of preparing the suspensions of the tissues are described and discussed with the results obtained, since they form an essential aspect of the problem investigated.

### *Results*

Rabbits were used as experimental animals. The compound was always freshly prepared and injected intravenously into the rabbit's ear. Mazur and Bodansky found that 1 mg. of DFP per kilo of rabbit applied intravenously is lethal. We are able to confirm this observation. If, however, 0.3 mg. of DFP per kilo is injected, the animals show increased reflex activity, muscle tremor, and sometimes convulsions, but most of them survive. According to Mazur and Bodansky, only about 10 per cent of the rabbits into which this dose is injected die. This is in satisfactory agreement with our own observation that out of forty injected rabbits, seven died, which is 17.5 per cent. The dose of 0.3 mg. per kilo may therefore be considered as close to the threshold which may become fatal.

*Determination of Cholinesterase in Tissue*—If the activity of cholinesterase present in nerve or muscle tissue has to be determined, the tissue has to be ground with great care. The suspension has to be completely homogeneous if a reliable quantitative evaluation of cholinesterase activity is desired, as has been emphasized previously. In case the method described by Potter and Elvehjem (13) is used, silicate has to be added, especially to the soft brain tissue, since otherwise the cell structure may not be destroyed, and the activity may differ considerably in aliquot parts of the homogenized suspension.

Previous observations have shown that the whole suspension has to be

used if the total cholinesterase activity is to be determined, since only a part of the enzyme present goes into solution. In all previous investigations the whole suspension was therefore used whenever the question of the total enzyme activity was involved. A few experiments were carried out again in order to test the distribution of the enzyme between precipitate and supernatant fluid.

A variety of nerve and muscle tissue of cat, rabbit, rat, and frog was ground in the homogenizer described by Potter and Elvehjem (13), the suspension was centrifuged, and the enzyme activity was determined separately in the precipitate and in the supernatant. Usually 80 to 90 per cent of the enzyme was found in the precipitate. The supernatant had negligible and sometimes even no enzyme activity. In only one case, 42 per cent of the enzyme activity was found in the supernatant.

The method of homogenization described by Potter and Elvehjem is most useful for preparations of small amounts of tissue up to about 0.5 gm. If a suspension of several gm. of tissue has to be prepared, the use of the Waring blender appears to be preferable. Extraction by this method seems to yield higher amounts of enzyme in the supernatant than by that of Potter and Elvehjem. About 35 to 45 per cent of the whole suspension prepared from rabbit brain and muscle was found in the supernatant. But as this is still less than half of the total enzyme activity, this method too requires use of the whole suspension when the question of the total enzyme activity present in a tissue is investigated.

*Cholinesterase in Normal Rabbit Brain*—The concentration of cholinesterase varies considerably in the different parts of the brain and in the different species. But it is remarkably constant for each species and for each brain section. It appeared preferable to determine the activity in small fragments of the brain for two reasons. First, in this way, several controls are possible in a single experiment. A second consideration is still more important. Previous observations have shown that after exposure to DFP small amounts of the compound are always present in the tissue. If a piece of tissue after exposure to DFP, or taken from an animal to which the compound has been injected, is ground in a cholinesterase solution, an inhibition is observed which depends on a variety of factors like weight of the tissue, amount of DFP used, length of exposure, etc. (9, 14). This inhibition has always to be taken into account. If the activity of cholinesterase in a tissue after exposure to DFP is determined, a parallel determination of the percentage inhibition due to the DFP still present and active has to be carried out on a corresponding piece of tissue of equal weight in order to correct the value obtained. Independently of any theoretical interpretation of the potential activity in the living tissue, it is obvious that the DFP present will inhibit the enzyme activity whether a solution con-

taining the enzyme is added or whether the enzyme is present in the tissue ground but was not previously inactivated by the DFP. The correction for the inhibition by the retained DFP is therefore a necessity for the correct estimation of the enzyme activity. It is obviously desirable to keep this inhibition at a low level. The smaller the amount of tissue used, the smaller will be the correction. Moreover, since the DFP has a high lipid solubility, a greater retention of the compound in the myelin appears probable and the use of gray matter therefore preferable.

TABLE I  
*Cholinesterase Activity in Brain Tissue of Normal Rabbits*

Cortex		Nucleus caudatus	
Weight	Acetylcholine split	Weight used	Acetylcholine split
mg.	mg. per gm. per hr.	mg.	mg. per gm. per hr.
58.0	49.0	11.5	283
53.0	46.5	11.4	258
52.0	54.0	11.6	420
58.8	53.0	11.0	390
57.3	33.8	10.7	322
56.3	39.5	10.6	328
53.4	36.5	10.0	238
53.7	52.0	11.5	366
52.3	44.8	12.5	302
57.0	39.0	11.6	396
53.8	59.0	12.3	236
58.5	32.0	11.5	255
57.5	46.5	10.5	300
54.3	33.2	9.4	237
54.5	55.5	12.1	389
		13.9	330
Average.....	45.0		315.6

Two sections of the brain were used in order to test the cholinesterase activity in the normal rabbit brain, the cortex and the nucleus caudatus. Both sections are easily obtained and well defined. The nucleus caudatus, moreover, has the advantage of having an unusually high concentration of cholinesterase (3). The data obtained are summarized in Table I. The average value for the cortex is 45 mg. of acetylcholine split per gm. per hour. The average value for the nucleus caudatus is 315.6 mg. of acetylcholine split per gm. per hour.

*Effect of DFP Injection on Brain Cholinesterase*—In a few experiments, 1 mg. of DFP per kilo was injected. The animals died within 1 to 2 minutes. In this case, the enzyme activity tested was zero or close to zero.

The cholinesterase activity was also determined in a few animals in which 0.3 mg. of DFP per kilo was injected and which died. In these cases, the cholinesterase concentration was always found to be very low, 2 to 4 per cent of the initial value at most.

If, however, the animals survived the injection of 0.3 mg. of DFP, cholinesterase was always found to be present in considerable amounts, although the percentage varied over a wide range. This is not surprising in such types of experiments in which so many factors are involved which may vary from one individual to the other. The data obtained are summarized in Table II. The lowest values were about 10 per cent of the normal average,

TABLE II

*Cholinesterase Activity in Brain Tissue of Rabbits Killed 25 Minutes after Injection of 0.3 Mg. of DFP per Kilo*

Experiment No.	Cortex			Nucleus caudatus		
	Weight used	Acetylcholine split		Weight used	Acetylcholine split	
		Uncorrected	Corrected		Uncorrected	Corrected
	mg.	mg. per gm. per hr.	mg. per gm. per hr.	mg.	mg. per gm. per hr.	mg. per gm. per hr.
1	91.0	9.5	13.7			
2	84.4	5.8	7.5			
3	83.8	13.0	22.5	32.0	82.0	82.0
4	76.0	8.5	11.4	39.5	45.0	55.0
5	78.2	3.6	4.6	39.8	38.0	55.0
6	87.5	8.5	12.1			
7	91.0	7.1	11.8	41.0	53.5	117.5
8	90.6	5.6	7.9	28.8	25.1	33.7
9				36.6	45.0	79.0
10	84.0	13.4	23.3			
11	82.2	3.5	4.5			
Average .....			11.9			70.4
% of normal average .....			26.4			22.3

but a few were close to 50 per cent. The average value of ten experiments with the cortex is 26.4 per cent of the normal average and in those experiments with the nucleus caudatus, 22.3 per cent of the normal average.

In all experiments, a slice of brain taken from the same section and of a weight equal to that used for cholinesterase determination was taken for the determination of the percentage inhibition of cholinesterase by the DFP retained. The piece of tissue was ground in a solution of brain esterase and, in this way, the percentage inhibition was obtained. The inhibition was not high, usually less than 30 per cent but varied from 20 to 40 per cent. The figures given in Table I are already corrected for this inhibition.



Since Mazur and Bodansky had obtained their results using the whole brain, it appeared interesting to compare the data obtained by the two methods. Four experiments were carried out with the whole brain. The amount of acetylcholine split per gm. per hour in the four experiments was rather constantly 1.5 to 2 mg. in the supernatant and 3.5 to 4 mg. in the whole suspension. This amounts to only 3 to 5 per cent of the normal activity. One possible explanation of these lower values may be a greater inhibition due to the larger amounts used and the larger proportion of myelin present in the whole brain than in thin slices of cortex or nucleus caudatus.

TABLE III

*Retention of DFP in Rabbit Brain Following Injection of DFP, 0.3 Mg. per Kilo*

Two corresponding pieces of brain tissue of the right and left hemisphere were ground, one in a cholinesterase solution prepared from rabbit brain, the other in Ringer's solution. The decrease of the CO<sub>2</sub> output indicates the inhibition by the DFP retained in the tissue.

Tissue	Weight	Medium of grinding	CO <sub>2</sub> output		Inhibition
			Observed	Corrected	
	mg.		c.mm. per hr.	c.mm. per hr.	per cent
Test solution			461.0	461.0	
Midbrain	226	Ringer's solution	87.0		
"	245	Test solution	324.0	237.0	48.5
Cerebral hemisphere	189.0	Ringer's solution	45.0		
" "	180.0	Test solution	142.0	97.0	79.0
Test solution			485.0	485.0	
Midbrain	216	Ringer's solution	48.5		
"	207	Test solution	326.0	277.5	43.0
Cerebral hemisphere	213	Ringer's solution	47.5		
" "	219	Test solution	246.0	198.5	59.0

Two experiments were performed to test the inhibition by larger amounts of brain tissue with the same proportion of fluid as in the experiments with the whole brain. Corresponding pieces of the brain were ground either in Ringer's or in the test solution for the calculation of the inhibition. The cholinesterase activity still present in the brain as found in the Ringer's solution has to be subtracted from the CO<sub>2</sub> output observed with the test solution in which the other pieces of brain tissue were ground. Two pieces of the cerebral hemisphere were used with a considerable amount of white matter attached and two pieces containing the corpora quadrigemina with some adjoining tissue. The results are given in Table III. The data indicate that the inhibition by the DFP retained is higher than that observed when smaller amounts of tissue and only gray matter are used.

## DISCUSSION

The experiments show, in agreement with previous observations (11), that injection of 1 mg. of DFP per kilo into a rabbit results in death within a few minutes. In these animals the cholinesterase of the brain tissue is completely inactivated. At the threshold dose of 0.3 mg. of DFP per kilo, negligible amounts of cholinesterase or none is found in the brain of those animals which die. But in the brain of the surviving rabbits, cholinesterase is always present. The concentration may be as low as 10 per cent of the initial, but the average is close to 25 per cent.

It has been established, in a great variety of experiments, that nerve conduction is inseparably associated with cholinesterase activity. The experiments reported here do not correlate directly cholinesterase activity and conduction as did the previous observations, but they show that the enzyme has a vital rôle in the function of the central nervous system and that its inactivation is incompatible with life. Not in a single animal which survived was the cholinesterase in the brain found to be absent. If it were possible for the animal to survive in complete absence of cholinesterase, it would be difficult to maintain the proposed rôle of acetylcholine in conduction, since life without conduction is impossible. The death resulting from injection of such small amounts of the compound has to be attributed to this specific and powerful action, since absence of the enzyme activity coincides always with death. In view of its high specificity the DFP effect is an excellent illustration of the idea so vigorously promoted and demonstrated by Peters; *viz*, that the effects of substances acting in very low concentrations may be often referred to an action on a single enzyme and that a complex biological effect may be traced to a particular and well defined chemical lesion.

Our data disagree with the observation of Mazur and Bodansky (11) that animals may survive in complete absence of cholinesterase and their conclusion that the presence of cholinesterase is fortuitous. The discrepancy is apparently due to the difference of the methods used. One possible factor in explaining the higher values obtained if only small amounts of tissue are used is a smaller inhibition by the DFP retained in the tissue. The smaller the amount of tissue used per vessel, the smaller will be the DFP concentration and the higher the activity actually observed. Moreover, the DFP may be retained in higher concentrations in sections rich in myelin and, if the whole brain is used, a stronger depression of the activity may result. Another factor which may contribute to the discrepancy, at least in some cases, is the use of supernatant fluids for the determination of the enzyme activity.

The necessity of cholinesterase has also been questioned by Riker and

Wescoc (15). They used for the cholinesterase determinations the supernatant fluid obtained by centrifugation of homogenized muscle suspensions. The figures obtained are only 10 to 30 per cent of the real total in normal cat muscle. The method used is therefore not adequate.

The percentage of cholinesterase found in the rabbits surviving the DFP injection requires some comments. Neither the average of 25 per cent nor the lowest values of 10 per cent should be used as indication for the minimum cholinesterase concentration necessary for normal functioning, or in other words, for the excess of enzyme available. It has been observed that at synaptic regions transmission is blocked at a much lower concentration of DFP than that required for abolition of axonal conduction (16). This interesting finding is, however, not surprising. All axons, including the so called unmyelinated, are surrounded by a lipid layer which acts as a protecting barrier. Only a small fraction of the outside concentration of DFP was found to penetrate through this barrier into the cell interior (14). At synapses there is no lipid layer and a part of the active surface is unprotected and therefore a lower DFP concentration is sufficient to inactivate the enzyme present in the pre- and postsynaptic membrane. Some vital centers may be easily accessible to the DFP and the concentration there may fall to very low values. If this inactivation is incompatible with life, death may occur at a period when the enzyme in other sections is still far in excess. Thus, a high average value may be found.

Experiments on the whole animal, however, in which so many functions and centers of different sensitivity are involved, are not favorable for deciding the question of excess. In experiments on the isolated frog sciatic nerve, it was found that up to 92 per cent of the initial cholinesterase activity can be abolished before conduction is impaired (unpublished data). In that case, the normal enzyme activity is 12 to 13 times in excess of the necessary minimum. This is not unusual, judging from the experience with other enzyme systems. For the evaluation of the remaining activity, the following thermodynamic consideration is essential.

When a single isolated impulse travels along a frog sciatic nerve at 20°, the initial heat averages about  $7 \times 10^{-8}$  gm. calorie or about 3 ergs per gm. of nerve (17). When a great number of impulses passes through the nerve, the initial heat per impulse decreases. At 30°, at a rate of 1000 impulses per second, the initial heat is about 0.5 erg. At 23°, at a rate of 300 to 400 impulses per second, about 2 to  $3 \times 10^{-8}$  gm. calorie is released as initial heat per gm. of nerve per impulse. If we assume that acetylcholine acts as a trigger and that 20 per cent of the initial heat may be attributed solely to the splitting of the ester, the heat for which acetylcholine hydrolysis would have to account would be 4 to  $6 \times 10^{-9}$  gm. calorie. On the basis of 2000 to 3000 gm. calories per mole released by the hydrolysis of acetylcholine,

this would be equivalent to about 0.0003 to 0.0004  $\gamma$  of acetylcholine. If this is the amount actually released and 1 to 1.5 million impulses pass through the frog sciatic per hour, 1 gm. of tissue should be able to remove 300 to 500  $\gamma$  of acetylcholine per hour. This is about 6 to 10 per cent of the amount which can be split in a normal frog sciatic nerve. Although for some assumptions the data are only approximations, the order of magnitude cannot be far off and the cholinesterase activity remaining after removal of the excess is consistent with that to be expected from the thermodynamic data available.

#### SUMMARY

The question whether the highly toxic effect of diisopropyl fluorophosphate (DFP) injected intravenously into rabbits has to be attributed to its action on cholinesterase has been investigated.

1. At a dose of 1 mg. of DFP per kilo injected intravenously, the animals die within a few minutes. The cholinesterase in the brain which is normally present in high concentration, splitting 80 mg. of acetylcholine per gm. per hour, is completely inactivated.

2. At a dose of 0.3 mg. of DFP per kilo, some animals die and others survive. In those which died, insignificant amounts of cholinesterase were found, or none. In all surviving animals, cholinesterase was always present without a single exception. The enzyme concentration varied between 10 and 50 per cent of the normal, averaging about 25 per cent.

3. Small amounts of tissue were used, reducing the inhibitory effect of the DFP retained in the tissue and liberated on grinding. Previously reported results which differ in some respects from those reported here were obtained by a different procedure on whole brains. In this connection, some methodological aspects of the problem are discussed.

4. In view of the high specificity of the action of DFP and its powerful effect on cholinesterase in low concentration, the coincidence of death and inactivation of cholinesterase suggest that the toxicity of the compound is due to its action on the enzyme.

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# A SPECIFIC COLOR REACTION FOR GLUCURONIC ACID\*

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The present report deals with a specific color reaction of glucuronic acid which can be used for the tentative identification in polyuronides. This reaction seems to be highly characteristic for glucuronic acid. It represents a specific form of a new general reaction of carbohydrates with SH compounds in  $H_2SO_4$  which differentiates various classes of carbohydrate<sub>s</sub> (methylpentoses, pentoses, hexoses, hexuronic acids) and individual hexose<sub>s</sub> and hexuronic acids.<sup>1</sup>

## EXPERIMENTAL

### *Reaction of Glucuronic Acid with Thioglycolic Acid in Presence of Mannose; Simple Qualitative Test*

*Procedure*—0.2 cc. of a 0.2 per cent solution of mannose is added to 0.8 cc. of a solution containing 0.02 to 0.04 per cent glucuronic acid. To this mixture are added with cooling in ice water and shaking 4.5 cc. of a water-sulfuric acid mixture prepared by mixing 6 volumes of concentrated  $H_2SO_4$  with 1 volume of  $H_2O$ . After 2 minutes in ice water the reaction mixture is placed in water of 20–25° and later for 3 minutes in boiling water. It is then cooled to room temperature and 0.1 cc. of a 2.5 per cent solution of thioglycolic acid (commercial preparation, solution prepared every few days and kept on ice) is added. The mixture is shaken thoroughly and left at room temperature for 20 to 24 hours. A deep pink color develops. The blank prepared with a mixture of 0.2 cc. of the mannose solution and 0.8 cc. of  $H_2O$  shows a greenish yellow color.

*Two Components of Color Reaction and Their Specificity*—The color reaction of glucuronic acid is the sum of two different reactions: (1) a reaction of glucuronic acid with thioglycolic acid alone and (2) a reaction in which all three reactants, mannose, thioglycolic acid, and glucuronic acid, participate. The first will be called the thioglycolic acid reaction, the second the mannose-thioglycolic acid reaction, designated by the abbreviations ThR and MThR respectively.

ThR can be observed separately by adding 0.2 cc. of  $H_2O$  instead of mannose solution to 0.8 cc. of the solution of glucuronic acid and treating

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<sup>1</sup> Dische, Z., *Federation Proc.*, 6, 248 (1947).

the sample in the same way as described above. The corresponding blank contains 1 cc. of water. Under these conditions glucuronic acid shows a brown-red color in lower concentration and a pure brown at 0.04 per cent. These colors are not characteristic for glucuronic but for hexuronic acids in general. Pentoses show an intensive pink, methylpentoses a green-yellow, and glucose and galactose a green and green-blue color respectively. The pink color observed in the presence of an excess of mannose, however, due to MThR, is completely specific for glucuronic acid and polyglucuronides. Galacturonic, pectic, and alginic acids and pneumococcus polysaccharide type I all show a non-characteristic brown color, and true sugars do not react with mannose at all. On the other hand not only mannose but all other hexoses react with glucuronic and thioglycolic acids with formation of a pink-colored product. These reactions, however, are not as specific as that with mannose.

#### *Spectrophotometric Test for Glucuronic Acid*

*Procedure for Measurement of Absorption Due to MThR*—In biological materials, we find in general glucuronic acid simultaneously with considerable amounts of true sugars. If the simple procedure described above is applied, the pink color of the ThR of pentoses may be mistaken for the MThR of glucuronic acid. Furthermore, when glucuronic acid is present in solution with a large excess of other hexuronic acids, the pink color of MThR may be covered by the intense brown color produced by the other compounds. In these cases, however, glucuronic acid still can be detected by spectrophotometric measurement of the intensity of MThR and with a slightly more elaborate procedure which consists of carrying out the reaction on two samples of the unknown solution (1) in the presence of mannose (MThR sample) and (2) in the absence of mannose (ThR sample). The absorption in the MThR sample gives the sum of the absorption due to MThR plus ThR (measured against a corresponding blank containing mannose); that in the ThR sample gives the absorption due to ThR (measured against a blank containing H<sub>2</sub>O only). The difference between the two gives the absorption due to MThR alone. This value will not, in general, be influenced by true sugars.

*Absorption Spectra for MThR of Various Hexuronic Acids and Polyuronides*<sup>2, 3</sup>—In Fig. 1 are recorded the absorption spectra of MThR of

<sup>2</sup> I am greatly indebted to Dr. Karl Meyer for preparations of hyaluronic and chondroitinsulfuric acids, to Dr. Michael Heidelberger for preparations of pneumococcus polysaccharides, and to Dr. Leonard Cretcher for a preparation of alginic acid.

<sup>3</sup> The content in hexuronic acids of the preparations of galacturonic, hyaluronic, and chondroitinsulfuric acids was determined by the modification of Freudenberg and his colleagues of the Lefèvre-Tollens method, that of pneumococcus polysaccharides by titration.

glucuronic, galacturonic, hyaluronic, chondroitinsulfuric, pectic, and alginic acids and of pneumococcus polysaccharide type I. The concentration of

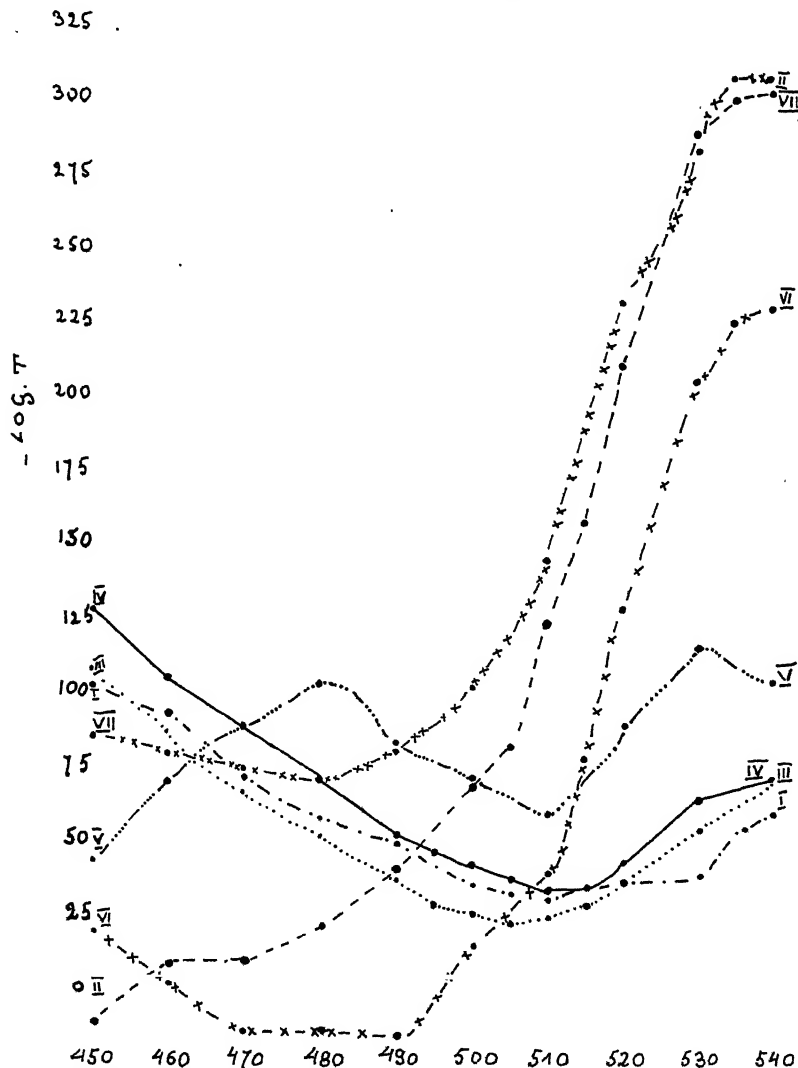


Fig. 1. Absorption spectra of MThR of some hexuronic acids and polyuronides. Curve I galacturonic acid, Curve II glucuronic acid, Curve III pneumococcus polysaccharide type I, Curve IV pectic acid, Curve V alginic acid, Curve VI hyaluronic acid, Curve VII chondroitinsulfuric acid. Wave-lengths in millimicrons ( $m\mu$ ).

the solutions was 0.01 per cent hexuronic acids and equivalent for the polyuronides. All spectra were measured in a Beckman spectrophotometer.



As can be seen, the pink color given by the glucuronic acid is due to a steep rise of the absorption starting at 5000A and reaching a maximum at 5400 A. The curves for hyaluronic and chondroitinsulfuric acids are almost identical in shape with that of glucuronic acid, though the absolute values for absorption are somewhat smaller for chondroitinsulfuric acid. This may be due to the fact that not all of the hexuronic acid in this polyuronide is glucuronic acid.

TABLE I

*Extinction Coefficients at 4800, 5100, and 5300 A and Their Differences for MThR of Hexuronic Acids and Polyuronides*

Readings after 22 hours; concentration of each hexuronic acid in solution 0.01 per cent.

Experiment No.	Substance	$E_{4800}$	$E_{5100}$	$E_{5300}$	$E_{5100} - E_{4800}$	$E_{5300} - E_{4800}$
I	Glucuronic acid	0.030	0.098	0.210	+0.068	+0.180
	Alginic acid	0.090	0.073	0.110	-0.017	+0.020
	Glucuronic + alginic acid	0.065	0.105	0.285	+0.040	+0.220
II	Glucuronic acid	0.060	0.180	0.310	+0.120	+0.250
	Galacturonic acid	0.055	0.055	0.080	0	+0.025
	Glucuronic plus galacturonic acid	0.055	0.170	0.365	+0.115	+0.310
III	Glucuronic acid	0.015	0.120	0.270	+0.105	+0.255
	Hyaluronic "	0.025	0.120	0.245	+0.095	+0.220
	Chondroitinsulfuric acid	0.035	0.115	0.225	+0.080	+0.190
	Alginic acid	0.280	0.260	0.300	-0.020	+0.020
IV	Glucuronic acid	0.085		0.240		+0.155
	Pectic acid	0.065	0.035	0.065	-0.030	0
	Pneumococcus polysaccharide type I	0.090	0.075	0.130	-0.015	+0.040
	Alginic acid	0.105	0.060	0.115	-0.045	+0.010
V	Glucuronic acid	0.150		0.400		+0.250
	Galacturonic acid	0.076		0.076		0
	Alginic acid	0.137		0.138		+0.001

The absorption curves for the other hexuronides and galacturonic acid are completely different. They show a flat minimum between 5000 and 5100 A.

*Absorption Increment between Two Wave-Lengths As Test for Glucuronic Acid*—The characteristic differences in the shape of the absorption curve between glucuronic acid and polyglucuronides, on the one hand, and other hexuronic acids and corresponding polyuronides, on the other, permit the detection of glucuronic acid, free or conjugated, in presence of an excess of other hexuronic acids. For this purpose we have only to measure the in-

tensity of MThR of the solution at 5100 and 4800 Å and subtract the second value from the first. This difference will be designated  $\Delta E_{5100-4800}$ . As can be seen from Table I this difference is positive for glucuronic acid and polyglucuronides and negative for the other hexuronic acids. Any positive value of  $\Delta E_{5100-4800}$  in an unknown solution indicates the presence of glucuronic acid. Positive values will be obtained even when glucuronic acid is present with a considerable excess of other hexuronic acids, because the negative values of  $\Delta E_{5100-4800}$  of other hexuronic acids are much smaller than the positive values for equivalent amounts of glucuronic acid.

If the purpose of the test is to exclude the presence of glucuronic acid in solution, it seems more convenient to determine the difference  $\Delta E_{5300-4800}$ . Table I shows this difference to be +0.012 for galacturonic acid (average of two determinations) and for alginic acid (average of four determinations) and zero for pectic acid (one determination). If  $\Delta E_{5300-4800}$  is not larger than 0.012, it can be assumed that no significant amounts of glucuronic acid are present in the solution.

*Influence of Hexuronic Acids and True Sugars on MThR of Glucuronic Acids*—When the concentration of every one of the hexuronic acids in solution does not exceed 0.01 per cent,  $\Delta E_{5100-4800}$  and  $\Delta E_{5300-4800}$  show no significant deviations from the additive behavior. When, however, the concentration of galacturonic or mannuronic acid exceeds that limit, the intensity of MThR of glucuronic acid is decreased and the test becomes less sensitive. It is therefore practical to work with solutions containing no more than about 0.01 per cent of total uronic acids.

True sugars also influence the intensity of MThR when their concentration exceeds certain limits; at 0.005 per cent galactose and arabinose are without influence, but glucose decreases MThR by about 20 per cent. The influence of glucose disappears at 0.002 per cent. A simple procedure for determining whether the concentration of hexoses in solution does not exceed this limit is the following.  $E_{5300} - E_{4800}$  is negative for the ThR of all hexuronic acids. When hexoses are present, the value of this difference is shifted towards the positive side. If after the addition of 0.01 cc. of glucuronic acid to the unknown solution,  $E_{5300} - E_{4800}$  of the ThR is not above zero, the concentration of hexoses in the solution is not high enough to influence MThR of glucuronic acid.

#### DISCUSSION

The validity of preliminary identifications based on color reaction always will appear subject to certain limitations. One of them is due in our case to the fact that the nature and mechanism of the reaction are unknown. It is, therefore, impossible to predict whether certain hexuronic or 5-ketohexonic acids or related compounds, so far not investigated, may not give

the reaction. So far only three hexuronic acids were found in nature, but others may be found in the future. Under these circumstances conclusions can safely be drawn only when our color reaction is negative. This indicates absence of glucuronic acid.

#### SUMMARY

1. A new characteristic color reaction of glucuronic acid is described.
2. This reaction permits the detection of glucuronic acid in free form or in polyuronides in presence of an excess of other hexuronic acids.

# CYSTINE AND RELATED COMPOUNDS IN THE NUTRITION OF LACTIC ACID BACTERIA\*

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The metabolism of certain inorganic forms of sulfur by various autotrophic bacteria has been studied extensively in the past. However, investigations dealing with the metabolism of sulfur compounds by the heterotrophic bacteria have not been numerous. Such work as has been done (reviewed by Meyers and Porter (1)) indicates that these microbial systems may be quite specific in their requirements for sulfur, especially for organic forms of sulfur.

It was not until the development of microbiological assays for amino acids that the exacting requirements of lactic acid bacteria for these nitrogenous substances became apparent. Indications that cystine might be required by several lactic acid bacteria were evident from the work of Wood, Geiger, and Werkman (2) in 1940. Later, Hutchings and Peterson (3) showed that cystine is required in a complete medium for *Lactobacillus casei* *s. e.* Shankman, Dunn, and Rubin (4), however, reported that they could not obtain reproducible standard curves for cystine with this organism. Shankman (5) and Hegsted (6) demonstrated the requirement for cystine by *L. arabinosus* 17-5; assays for cystine with this organism have been proposed by Shankman, Dunn, and Rubin (4) and by Barton-Wright (7). The absolute requirement of cystine by *L. arabinosus*, however, could not be confirmed in this laboratory (unpublished data). According to Dunn *et al.* (8), cystine is required by *Leuconostoc mesenteroides* PD-60 but was found to be unessential for *Lactobacillus fermenti* (9). In experiments by Greenhut, Schweigert, and Elvehjem (10), *Streptococcus faecalis* R grew only slightly on a chemically defined medium free from cystine; later work in this laboratory indicates that cystine is merely stimulatory to the growth of this organism.

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Since the cultures of *Lactobacillus arabinosus* 17-5 which were available did not require cystine, the possibility of developing an assay method for cystine appeared to need further investigation. A number of the previously mentioned organisms were selected to test their requirements for cystine. This necessitated the development of suitable media adequate for the organism used, yet free from this amino acid.

Attempts were also made to test the ability of a number of organic and inorganic sulfur-containing compounds to replace cystine in the media for organisms which require cystine. Previous work had indicated that heat treatment alters the availability of cystine for bacteria; the effect upon the compounds tested of sterilization by autoclaving as compared to filtration was accordingly determined.

#### EXPERIMENTAL

*Microorganisms*—Eight microorganisms were used in these experiments: *Lactobacillus arabinosus* 17-5, *L. fermenti* 36, *L. casei*  $\epsilon$ , *L. pentosus*, *L. delbrueckii* LD-3, *L. delbrueckii* LD-5, *Streptococcus faecalis* R, and *Leuconostoc mesenteroides* PD-60. The following procedure was used in the preparation of all inocula. The original cultures of the organisms were maintained in the form of lyophilized cultures as described by Nymon, Gunsalus, and Gortner (11). All of the organisms used in this study have been found to be viable after a year of storage in this manner. Stock cultures were carried by weekly transfer to stabs containing a nutrient-rich medium (peptone 1 per cent, Difco-soluble yeast extract 0.5 per cent, glucose 2 per cent, vitamins B 2 ml. per cent,<sup>1</sup> Salts B 0.5 ml. per cent,<sup>1</sup> sodium acetate 1 per cent, potassium phosphate (dibasic) 0.5 ml. per cent,<sup>1</sup> and agar 2 per cent). A small amount of the material from the stab cultures was incubated for 12 hours at 37° in 10 ml. of a medium of the same composition (agar omitted). The cells were centrifuged and resuspended in 50 ml. of 0.9 per cent saline prior to inoculation of the tubes.

*Media*—In an attempt to obtain a medium free from cystine, three sources of amino acids were used: (1) crystalline amino acids (synthetic amino acids were used wherever possible to avoid possible cystine contamination), (2) an acid hydrolysate of casein oxidized with hydrogen peroxide (12, 13), and (3) peptone oxidized with hydrogen peroxide (14). It had been found previously that an acid hydrolysate of casein, such as is commonly used for tryptophan assay, was not low enough in cystine content to permit its use for assay purposes. In other respects, the media were similar to those previously used (12). The composition of the media as well as the method of designation used to refer to the various components of the media is presented in Table I.

<sup>1</sup> For concentrations of the stock solutions see Riesen, Schweigert, and Elvehjem (12).

TABLE I  
Composition of Basal Media\*

Amino acids, mg. per liter				
	S	C	P	
Acid hydrolysate of oxidized casein (15, 16)		5000	5000	
Oxidized peptone (17)				
L-Glutamic acid	400			
L-Asparagine	400			
L-Lysine monohydrochloride monohydrate	200			
DL-Threonine	200			
DL-Valine	200			
DL-Isoleucine	200			
DL-Alanine	200			
DL-Leucine	200			
DL-Phenylalanine	100			
L-Arginine hydrochloride	100			
L-Histidine " monohydrate	100			
DL-Methionine	200	200	200	
L-Tyrosine	100		100	
DL-Tryptophan	100	100		
DL-Serine	200			
DL-Proline	100	100		
Glycine	100		100	
Buffers, gm. per liter				
	a	am	c	cm
Sodium acetate	20	20		
" citrate monohydrate			25	25
Ammonium chloride		3		3
Salts, mg. per liter				
	AB	BP	CP	
KH <sub>2</sub> PO <sub>4</sub>	500		5000	
K <sub>2</sub> HPO <sub>4</sub>	500			
MgSO <sub>4</sub> ·7H <sub>2</sub> O	200	200	800	
NaCl	10	10	40	
FeSO <sub>4</sub> ·7H <sub>2</sub> O	10	10	40	
MnSO <sub>4</sub> ·4H <sub>2</sub> O	10	10	160	

\* The stock solutions were prepared as described by Riesen *et al.* (12). The basal media were prepared at twice the concentration of the constituents listed which represent the final tube concentration (total volume 2 ml.). The neutralized media were stored in 100 ml. quantities at -4°. All basal media are referred to in the text and in the figures by the abbreviations above; *e.g.*, S:a:AB for a medium consisting of crystalline amino acids, acetate buffer, and Salts A and B in addition to the remaining constituents of all media.

TABLE I—*Concluded.*

Constituents of all media, mg. per liter

Adenine sulfate dihydrate	10	Calcium <i>dl</i> -pantothenate	0.5
Guanine monohydrochloride dihydrate	10	Riboflavin	0.5
Uracil	10	Nicotinic acid	1.0
Xanthine	10	<i>p</i> -Aminobenzoic acid	0.1
Thiamine monohydrochloride	0.5	Biotin	1 $\gamma$
Pyridoxamine dihydrochloride	0.2	Folic acid	10 $\gamma$
		Glucose	20 gm.

*Assay Procedure*—All assays were conducted on a semimicro scale in which the total tube volume was 2 ml. The solutions of standards or of compounds to be tested (0, 0.1, 0.2, 0.4, 0.6, 0.8 ml.), medium (1 ml.), and water (sufficient to make a total of 2 ml.) were added with a Cannon automatic dispenser<sup>2</sup> to 18  $\times$  50 mm. Pyrex culture tubes in racks containing six tubes per row. The racks, which were provided with metal covers fitted with cotton pads, were autoclaved for 15 minutes at 15 pounds pressure and the tubes inoculated with 1 drop from the diluted saline suspension of cells and incubated at 37° for 3 days. The acid produced was titrated electrometrically directly in the tubes with a 5 ml. micro burette equipped with a titration device and a built in air stirrer,<sup>3</sup> together with a quinhydrone electrode assembly.

*Treatment of Compounds Tested*—L-Cystine (Merck) was used as the standard for the assays. The following compounds were tested for cystine activity: L-cystine hydrochloride, DL-cystine, *meso*-cystine, glutathione, DL-homocystine, sodium thioglycolate, amyl mercaptan, sodium sulfide monohydrate, and sodium thiosulfate pentahydrate. The sulfur-containing amino acids were maintained in stock solution at a concentration of 1 mg. per ml. under toluene in dark bottles in the refrigerator. Solutions of all other compounds were prepared prior to each assay. The final concentrations of the compounds tested were such that each ml. contained the same amount of sulfur as did 1 ml. of the standard L-cystine. This permitted a direct comparison of the acid produced by the compound being tested with that produced by cystine. DL Compounds including *meso*-cystine were doubled in concentration; thus when the DL compound gives the same acid production as L-cystine, the D isomer has no activity.

Measurements were also made of the cystine activity of glutathione after it had been hydrolyzed with acid or by enzymes. For acid hydrolysis, 12.8

<sup>2</sup> Cannon, M. D., *et al.*, to be published.

<sup>3</sup> Private communication with Dr. F. M. Strong of the Department of Biochemistry of the University of Wisconsin.

ml. of a solution containing 1 mg. of glutathione per ml. were autoclaved with 2 ml. of 2 N HCl for 2 hours at 15 pounds pressure. The enzyme hydrolysates were prepared by shaking 12.8 ml. of glutathione (1 mg. per ml.) with 2 mg. of Merck pancreatin, 1 mg. of Wilson's crude hog intestinal mucosa, 50 ml. of pH 8.6 sodium carbonate buffer, and 1 ml. of toluene for 3 days at 37°. These enzyme preparations had been tested previously for crude proteinase and peptidase activity.

The compounds tested were sterilized by autoclaving them with the media, or by filtering them through sintered glass bacterial filters prior to aseptically dispensing into autoclaved media.

The effect of a reducing medium upon the activity of a number of the compounds tested was investigated by autoclaving media with 0.5 mg. of ascorbic acid or 0.5 mg. of sodium thioglycolate added per assay tube. The latter compound could obviously be tested only with microorganisms for which it could not replace cystine in an otherwise complete medium.

#### RESULTS AND DISCUSSION

*Requirements*—It can be seen from Table II that the media containing three different sources of amino acids were sufficiently low in cystine to permit their use in the microbiological assay of this amino acid. With cystine present, the oxidized peptone media gave the highest acid production with most organisms (see Fig. 1 for the *Lactobacillus pentosus* curves). Media containing an acid hydrolysate of oxidized casein gave intermediate acid production, while crystalline amino acid media gave the least. It is possible that unknown amino acids or peptides which stimulate the growth of microorganisms exist in the casein and peptone media.

Probable contamination of amino acids with cystine is indicated by higher blank titrations obtained with synthetic media than with media containing oxidized casein or peptone (Table II). Some commercial samples of L-leucine have been found to be contaminated with methionine (12), and Barton-Wright (7) has reported the occurrence of cystine in commercial samples of L-tyrosine.

Table II shows the growth obtained in terms of acid production when cystine was omitted from the media for each microorganism used in this study. Cystine was found to be required by *Lactobacillus casei*, *L. pentosus*, *L. delbrueckii* 3, and *Leuconostoc mesenteroides*; it stimulated the growth of *L. arabinosus*, *L. delbrueckii* 5, and *Streptococcus faecalis* R somewhat, while it proved to be unessential for the growth of *Lactobacillus fermenti*. Other workers have found that cystine is required in media for *S. faecalis* R and *L. arabinosus*. These differences may be due to the use of media which are not entirely free of cystine, or to different strains of the same organism used in different laboratories. However, since the essential nature



of cystine for the four above named microorganisms was demonstrated with three different sources of amino acids, it would seem that differences to be found in the literature with those reported here are due to differences in the

TABLE II  
*L-Cystine Requirement of Various Lactic Acid Bacteria\**

Organism	Basal medium†	Titer, cystine omitted‡	Concentration at half maximum growth§	Requirement shown by others in literature
		ml. 0.05 N NaOH	$\gamma$ per tube	
<i>L. arabinosus</i> 17-5	S:a:AB	3.10±		+ (5-7)
	C:a:AB	4.00±		
	P:a:AB	5.90±		
" <i>fermenti</i> 36	S:a:AB	3.70-		- (9)
	C:a:AB	3.20-		
	P:a:AB	3.20-		
" <i>casei</i>	S:c:CP	0.80+	50	+ (3)
	C:c:CP	0.60+	40	
	P:c:CP	0.65+	35	
" <i>pentosus</i>	S:a:AB	0.35+	5	
	C:a:AB	0.30+	5	
	P:a:AB	0.30+	4	
" <i>delbrueckii</i> LD-3	S:a:AB	0.55+	5	
	C:a:AB	0.20+	4	
	P:a:AB	0.25+	4	
" " LD-5	S:a:AB	1.90±		
	C:a:AB	2.40±		
	P:a:AB	1.30±		
<i>Streptococcus faecalis</i> R	S:c:BP	3.00±		+ (10)
	C:c:BP	3.50±		
	P:c:BP	2.00±		
<i>Leuconostoc mesenteroides</i>	S:am:AB	0.70+	4	+ (8)
PD-60	C:am:AB	0.35+	4	
	P:am:AB	0.40+	3	

\* The requirement of cystine is indicated by a (+), stimulation with (±), and dispensability with (-).

† See Table I for the composition of the basal media corresponding to these abbreviations. All media were autoclaved as usual for sterilization.

‡ The maximum titrations were from 4 to 7 ml., depending upon the organism and the medium used. The titrations of uninoculated tubes were 0.2 ml. for media with acetate buffer and 0.6 ml. for media with citrate buffer.

§ The total tube volume was 2 ml.

strain. There is a great variation in the amount of cystine required by the four organisms for which it is essential. *L. casei* requires approximately 35, *L. delbrueckii* 3 approximately 4, *L. pentosus* 4, and *L. mesenteroides* 3  $\gamma$  per

tube for half maximum growth on the oxidized peptone medium. In several cases, the level of cystine which produced half maximum growth was higher with synthetic media and oxidized casein media than those in which oxidized peptone was used.

*Effect of Autoclaving*—In most routine assays, media are sterilized by autoclaving them at 12 to 15 pounds pressure for from 10 to 15 minutes, and most amino acids added prior to this treatment can be recovered satisfactorily. Nevertheless, preliminary experiments indicated that cystine may

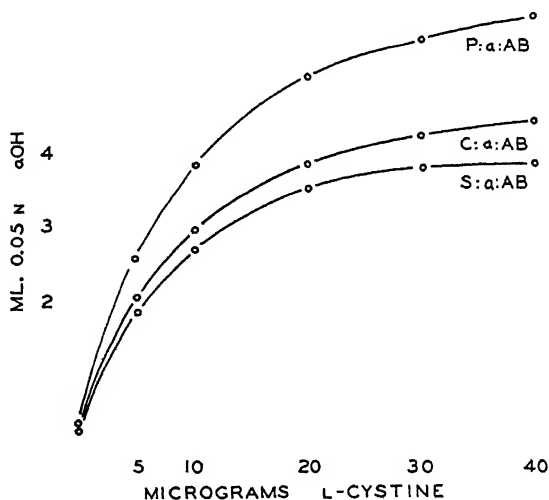


FIG. 1. Cystine (autoclaved) standard curves obtained with *L. pentosus* with media containing oxidized peptone, acid-hydrolyzed oxidized casein, or crystalline amino acids. See Table I for the composition of media corresponding to the abbreviations used.

be destroyed or modified in the autoclave and that a satisfactory "recovery" of cystine does not necessarily prove that no loss has taken place.

To test this possibility further, standard curves from assays in which cystine had been autoclaved with the medium were compared with standard curves of cystine which had been filtered with a bacterial sintered glass filter prior to aseptic addition to tubes containing autoclaved medium. This procedure was also used to test the effect of autoclaving upon other compounds which were assayed for cystine activity. In each case, the final tube contained the same amount of sulfur as that supplied by L-cystine. Racemic compounds and the internally compensated *meso*-cystine contained twice as much sulfur per tube as tubes containing L-cystine. The compounds were filtered after final dilution to the same concentration used for

standard curves of the autoclaved compounds. The activities of the compounds subjected to each method of sterilization were then expressed by the slope of the most nearly linear (usually the lower portion) part of the standard curves obtained with any particular organism divided by the slope of filtered L-cystine for the same organism multiplied by 100. These results are summarized in Table III and in Figs. 2 to 12.

The results obtained indicate that autoclaving resulted in reduction of cystine activity to 44 to 48 per cent when tested with *Leuconostoc mesenteroides*, to 23 to 29 per cent when tested with *Lactobacillus delbrueckii* 3, to 39 to 43 per cent with *L. pentosus*. Little or no loss was observed if *L. casei* was used. Similar results were obtained whether the non-cystine nitrogenous source was crystalline amino acids or oxidized peptone. The addition of reducing agents, such as sodium thioglycolate or ascorbic acid, had no effect upon the destruction caused by autoclaving. Similar results were also obtained with L-cysteine.

It is well known that cystine is rapidly destroyed when heated in an alkaline medium, and work in this laboratory indicates that 20 to 60 per cent of cystine may be inactivated when autoclaved with 2 N acid at 15 pounds pressure for 3 to 12 hours. Present results indicate inactivation as tested with microorganisms when cystine is heated at pH 6.8 for relatively short periods of time. The extent of inactivation, however, did not appear to be the same for each microorganism. Thus cystine modified by being autoclaved was fully as active as filtered cystine for *Lactobacillus casei*. Since most of the cystine activity was lost when tested with *Lactobacillus delbrueckii* 3, it is apparent that a large percentage of the cystine present underwent modification. Although there is no concrete evidence which indicates the nature of this change, cystine is utilized by the organisms tested not only as cystine *per se* but also to a varying degree as modified cystine.

There is evidence that a reaction occurs between cystine and remaining components of the medium with which it is autoclaved. It was noted that assay tubes in which filtered cystine was incorporated after autoclaving of the cystine-free medium appeared darker brown in color than tubes in which cystine was autoclaved with the medium. In all cases, tubes were never autoclaved longer than 15 minutes at 15 pounds pressure. This observation may be associated with the loss in activity of cystine noted above.

*Activity of Related Compounds; Lactobacillus casei*—The comparative activities of L-cystine, L-cysteine, DL-cystine, and meso-cystine for *Lactobacillus casei* are shown in Fig. 2 and Table III. It is of interest to note the rather high levels of cystine (25 to 50  $\gamma$  per tube for half maximum growth) required by this organism. L-Cystine and L-cysteine are nearly equivalent in their activity in the lower portions of the growth curves. However, the filtered compounds showed inhibitory tendencies at higher levels; these were

TABLE III

Summary of Cystine Activity of Sulfur-Containing Compounds for Lactic Acid Bacteria That Require Cystine\*

Compound	Sterilization†	<i>L. casei</i>	<i>L. pentosus</i>	<i>L. delbrueckii</i> 3	<i>L. mesenteroides</i>
L-Cystine	Filtered	(100)	(100)	(100)	(100)
	Autoclaved	100 P	43, 35, 49 P; 43 C	29, 23 P; 25 C	46, 47, 10‡ P; 48 C; 44 S
L-Cysteine + HCl	Filtered	100, 139 P	103, 96 P	100 P	98, 80 P; 101 S
	Autoclaved	100, 122 P	79, 43 P	28, 29 P	43, 63, 12‡ P; 45 S
DL-Cystine	Filtered	Inhibits	143 P	Inhibits	127 P
	Autoclaved	130 P	26 P	"	34 P
meso-Cystine	Filtered	85 P	187 P	"	168 P
	Autoclaved	164 P	29 P	"	48 P
Glutathione	Filtered	1300, 2000, 1500 P; 1300 S	196 P		2 P; 4 S
	Autoclaved	1400 P	220, 171, 164 P; 106 C	10, 11 P; 39 C	8, 10 P; 3 C
Glutathione, acid- hydrolyzed	Filtered	2200, 900 P	100 P	100 P	100, 105 P
	Autoclaved	200 P	48 P		48 P
Glutathione, en- zyme-treated	Filtered	500 P	250 P		50 P
	Autoclaved	1500 P	250 P		20 P
DL-Homocystine	Filtered	2 P	Activity at high levels (Fig. 7)	0, 2§ P	4, 10‡P; 0 S
	Autoclaved	28 P		3, 3, 8§ P	0, 3, 0, 5‡P; 0 S
Sodium thio- glycolate	"	100, 100 P	0, 2 P	5 P	0, 0 P‡
Amyl mercaptan	"	Slight P	0 P	Slight P	0 P
Sodium sulfide monohydrate	"	100, 78 P	0, 1 P	5 P	0 P
Sodium thiosulfate pentahydrate	"	55 P	0 P	Slight P	0 P

\* Activities are expressed in terms of acid production in a cystine-free medium when compared with an equivalent amount on the basis of sulfur content of L-cystine sterilized with a bacterial filter which was given an arbitrary activity of 100. The compounds containing molecular components having the D configuration were doubled in quantity. The letters following the figures refer to the source of amino acids used in the media (see Table I). Repeated figures refer to repeated assay.

† The compounds were sterilized either by filtration through a sintered glass filter or by autoclaving at 15 pounds pressure for 15 minutes. The media were autoclaved in all cases.

‡ Plus 0.5 mg. of sodium thioglycolate or ascorbic acid per tube.

§ Plus 0.5 mg. of ascorbic acid per tube.

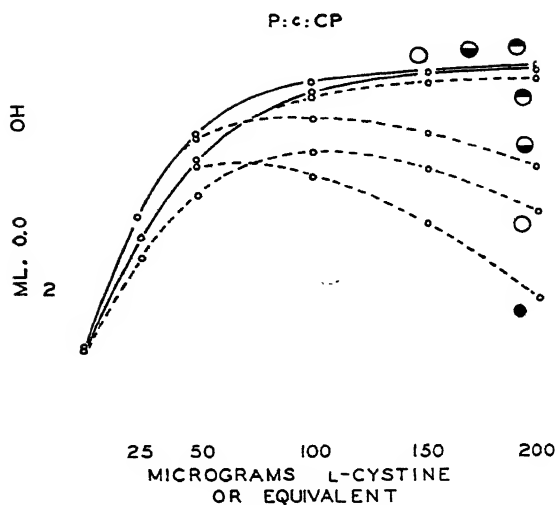


FIG. 2. Typical cystine standard curves and activity curves of related compounds for *L. casei* (P:c:CP); ●, L-cystine, ○ L-cysteine + HCl, ◐ DL-cystine, ◑ meso-cystine. The compounds represented in these and succeeding figures were sterilized by filtration (dash line) or by autoclaving (solid line) with the medium. The curves are plotted to permit direct comparison of the activity of each compound on the basis of its sulfur content. The media are defined in Table I.

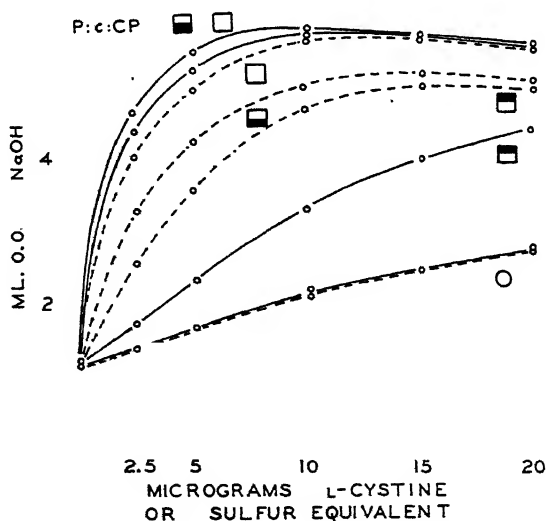


FIG. 3. See the legend for Fig. 2; ○ L-cystine + HCl, □ glutathione, ◐ glutathione, acid-hydrolyzed, ◑ glutathione, enzyme-treated.

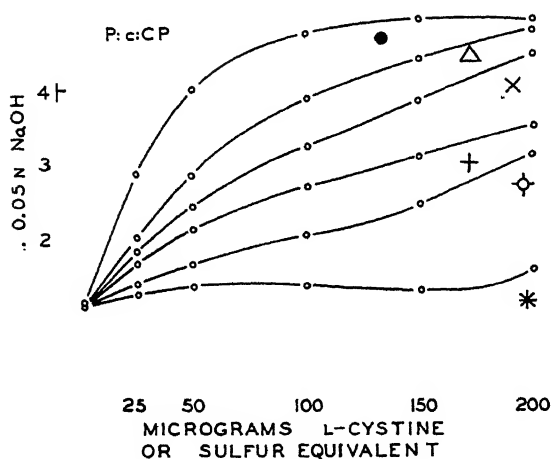


FIG. 4. See the legend for Fig. 2; ● L-cystine, ○ DL-homocystine, △ sodium thioglycolate, \* amyl mercaptan, × sodium sulfide +  $9\text{H}_2\text{O}$ , + sodium thiosulfate +  $5\text{H}_2\text{O}$ .

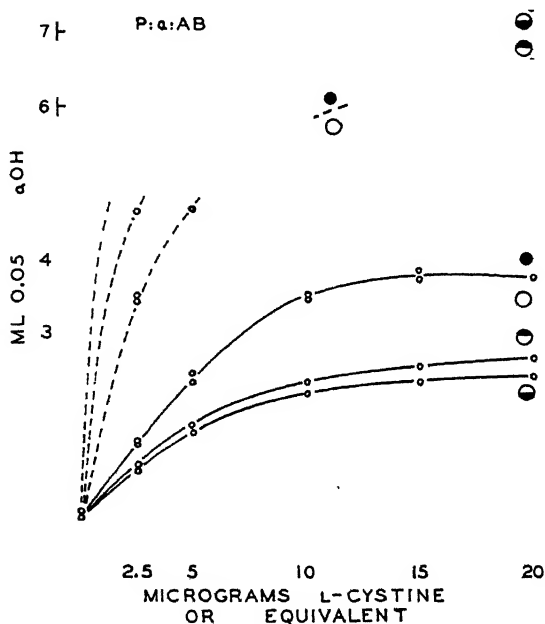


FIG. 5. Curves showing the activity of cystine and related compounds for *L. pentosus* (P: a: AB); ● L-cystine, ○ L-cystine + HCl, ◐ DL-cystine, ◑ meso-cystine. The media are defined in Table I.

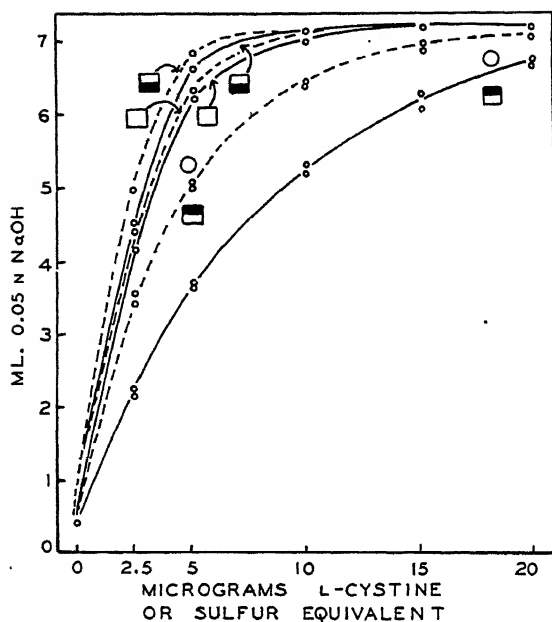


FIG. 6. See the legend for Fig. 5; ○ L-cystine + HCl, □ glutathione, glutathione, acid-hydrolyzed, ■ glutathione, enzyme-treated.

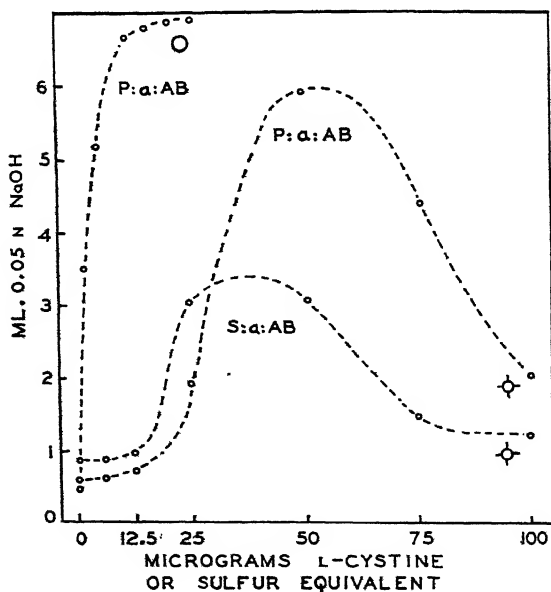


FIG. 7. See the legend for Fig. 5; ○ L-cystine + HCl, ◇ DL-homocystine

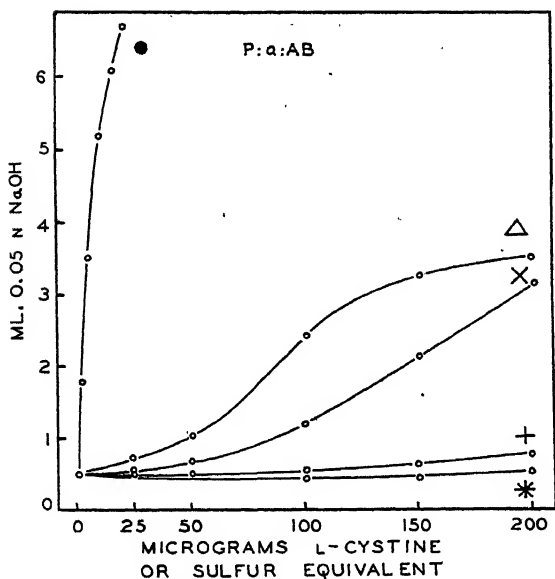


FIG. 8. See the legend for Fig. 5; ● L-cystine, △ sodium thioglycolate, \* amyl mercaptan, × sodium sulfide +  $9\text{H}_2\text{O}$ , + sodium thiosulfate +  $5\text{H}_2\text{O}$ .

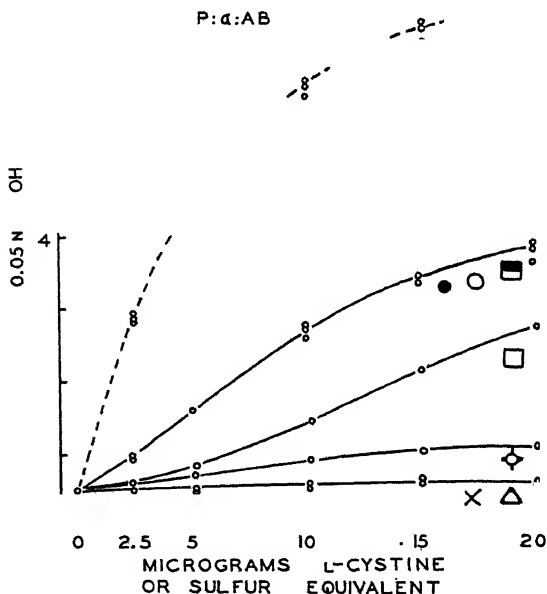


FIG. 9. Curves show the activity of cystine and related compounds for *L. delbrueckii* 3 (P:a:AB); ● L-cystine, ○ L-cysteine + HCl, □ glutathione, ■ glutathione, acid-hydrolyzed, ◇ DL-homocystine, △ sodium thioglycolate, × sodium sulfide +  $9\text{H}_2\text{O}$ .



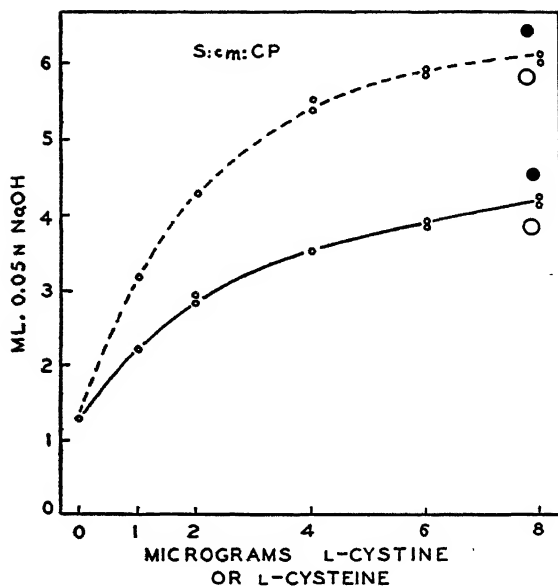


FIG. 10. Curves showing the activity of cystine and related compounds for *L. mesenteroides* (S:cm:CP) (solid line, autoclaved, dash line, filtered); ● L-cystine, ○ L-cysteine + HCl.

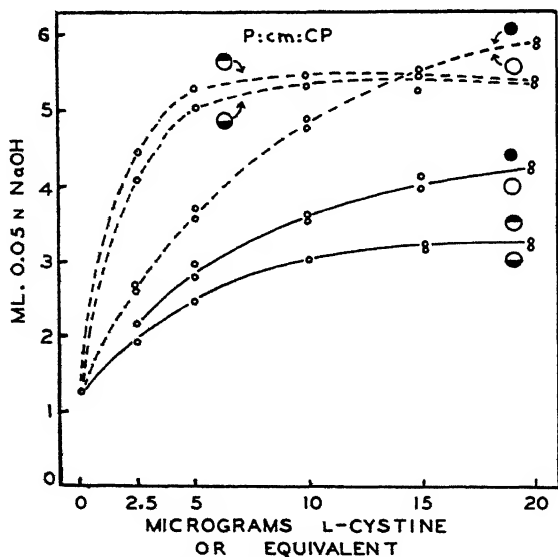


FIG. 11. See the legend for Fig. 10 (P:cm:CP); ● L-cystine, ○ L-cysteine + HCl, ● DL-cystine, ● meso-cystine.

overcome by autoclaving the compounds with the medium. Autoclaving also had beneficial effects in raising the activity of *meso*-cystine and DL-cystine toward that of L-cystine.

In Fig. 3, the activities of glutathione before and after hydrolysis are compared with that of L-cystine and L-cystine. In repeated assays, glutathione proved to be 13 to 20 times as active as cystine or cysteine. This result was unaffected by autoclaving the peptide at 15 pounds pressure for 15 minutes. In view of the sensitivity of glutathione to heat, it would appear that modified forms of this tripeptide are very active for *Lactobacillus casei*.

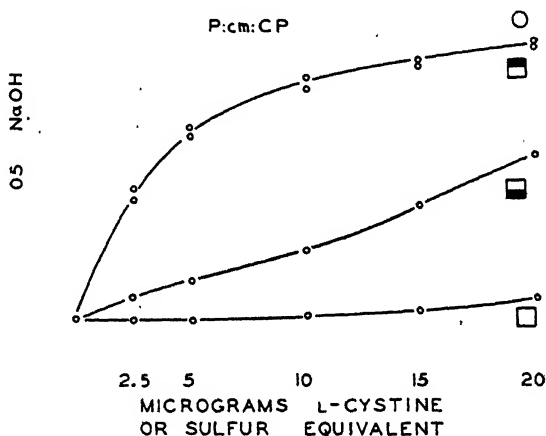


FIG. 12. See the legend for Fig. 10 (P:cm:CP); ○ L-cystine + HCl, □ glutathione, ■ glutathione, acid-hydrolyzed, ■ glutathione, enzyme-treated.

Scott, Norris, and Heuser (15) pointed out that glutathione increases the initial growth of *Lactobacillus casei* in an otherwise complete medium. These workers used a turbidimetric assay at the end of 16 hours of incubation at 37°. In the present work, all inoculated media were incubated for 72 hours at 37° and the growth was measured by titration of the lactic acid produced. The results shown in Fig. 3 indicate that glutathione is approximately 20 times as active as cystine itself for *L. casei*, and it is accordingly postulated that cystine is not the actual compound used by this organism for the synthesis of protein, but that glutathione *per se* more nearly meets its synthetic requirements for a cystine-like compound. Increased turbidity of inoculated media during incubation likewise indicates that glutathione is more readily utilized for cell production than cystine.

Partial hydrolysis products of glutathione retain the high activity orig-

inally possessed by the intact compound. An attempt to hydrolyze the compound with a mixture of pancreatin and hog intestinal mucosa (which were known to have good protease and peptidase activity) was unsuccessful in decreasing the activity to that of the parent amino acid cysteine for this organism; the activity was actually increased by the action of the crude enzyme mixture. Acid hydrolysis (which was known to hydrolyze glutathione when tested with other microorganisms) likewise affected the activity to a varying degree; occasionally the modified products were more active than the original glutathione.

It is possible that some of the difficulties reported with *Lactobacillus casei* in microbiological assays may be due to limiting amounts of cystine in the medium. Since relatively small amounts of glutathione effectively replace cystine, and since autoclaved glutathione also has high activity, this is an ideal compound to supply the sulfur requirements other than methionine for this microorganism. It is of interest to recall, in this connection, that Woolley (16) has reported partial streptogenin activity with *Lactobacillus casei* of a tripeptide (serylglycylglutamic acid) containing two amino acids found in glutathione. Apparently a number of peptides have stimulatory effects upon the growth of this microorganism.

Most of the remaining compounds tested (Fig. 4) replaced cystine in the following order of increasing activity: amyl mercaptan, DL-homocystine, sodium thiosulfate, sodium sulfide, and sodium thioglycolate (which had activity equal to that of L-cystine). It is evident that *Lactobacillus casei* can utilize many diverse forms of organic and inorganic sulfur as partial replacements for cystine in its nutritive medium.

*Lactobacillus pentosus*—Fig. 5 indicates that cystine suffered at least one-half loss in activity during autoclaving when tested with *Lactobacillus pentosus*. Cystine and cysteine were approximately equivalent in activity irrespective of the method of sterilization. Isomers containing unnatural components (DL-cystine and meso-cystine) gave standard curves in which the effect of autoclaving was a reduction in activity due to the D isomer. When the compounds were filtered, the D components had partial cystine activity. It seems that autoclaving modifies the D amino acid (whether it be D-cystine or D-cysteine from meso-cystine) to a different extent than the L isomer.

Fig. 6 shows that glutathione has somewhat greater activity for *Lactobacillus pentosus* than could be accounted for from its cysteine content. It is possible that this was accentuated by peptides present in the medium, since equivalent activity with cysteine was obtained with a medium containing oxidized casein. As was the case with *L. casei*, autoclaving had no effect in diminishing the activity of glutathione, and enzyme "hydrolysis" of this peptide likewise increased its activity for *L. pentosus*. However, acid hydrolysis resulted in a reduction in activity of the cleavage

products to that expected from cysteine. This was true both in the cases of autoclaved and filtered samples of the acid hydrolysate of glutathione.

Fig. 7 shows typical curves obtained with DL-homocystine for *Lactobacillus pentosus*. At low levels, this compound had little activity; however, at higher concentrations the activity rose sharply and finally reached a maximum equal to that obtained with high levels of cystine.

The addition of serine did not affect the rate of conversion; thus cystathionine is probably not the intermediate compound involved. It is interesting that similar curves have been obtained by Hac, Snell, and Williams (17) with *Lactobacillus arabinosus* when the conversion of glutamic acid to glutamine in the presence of ammonium salts was tested.

Sodium sulfide (Fig. 8) and sodium thioglycolate had negligible cystine activity. The remaining compounds tested, sodium thiosulfate and amyl mercaptan, possessed no cystine activity for *Lactobacillus pentosus*.

*Lactobacillus delbrueckii* 3—The activities of various compounds for this organism are summarized in Table III and are shown in Fig. 9. The destructive effects of autoclaving are again apparent in the case of cystine and cysteine, which are otherwise nearly equivalent in activity. The isomeric forms (DL-cystine and meso-cystine) usually inhibited the growth of this organism; however, the results were not uniformly reproducible. Glutathione possessed less activity than L-cystine in an oxidized peptone medium and more activity in an oxidized casein medium. Acid-hydrolyzed glutathione showed equivalent activity with filtered L-cystine and L-cysteine. Homocystine had partial activity which was accentuated by the addition of ascorbic acid. Sodium thioglycolate and sodium sulfide had only slight activity, and sodium thiosulfate was entirely devoid of cystine activity.

*Leuconostoc mesenteroides*—This organism (Fig. 10) responds to the smallest amount of cystine of any organism tested. Approximately 50 to 60 per cent of the cystine activity is lost during autoclaving. However, cystine and cysteine were found to be equivalent when filtered, and usually equivalent when autoclaved. These effects were the same with the medium containing crystalline amino acids as with a medium containing oxidized peptone. The addition of ascorbic acid had little effect upon the activity of these compounds; however, sodium thioglycolate (which was itself inactive) had inhibitory effects upon the acid production when present in the medium in addition to graded amounts of cystine. The cystine standard curves obtained with this organism were more reproducible than those obtained with any other strain of bacteria tested.

It can be seen from Fig. 11 that DL-cystine and meso-cystine inhibited the growth of *Leuconostoc mesenteroides* when the latter compounds were autoclaved with the medium. However, the D forms had partial activity when DL-cystine and meso-cystine were filtered.

Fig. 12 shows that intact glutathione has very little cystine activity for *Leuconostoc mesenteroides*. Enzyme hydrolysis resulted in partial activity of this compound; acid hydrolysis produced activity equal to that of the cleavage product cysteine. Since intact glutathione has no activity, the increasing activity during hydrolysis serves as an index of the relative efficiency of hydrolysis of this compound by enzymes and by acid.

All compounds tested other than cystine or cysteine possess practically no activity for this organism. *Leuconostoc mesenteroides* is the most suitable of any organism tested for the microbiological assay of cystine, since it is the most specific in its response to cystine as compared to other forms of this amino acid.

#### SUMMARY

The cystine requirement for eight microorganisms commonly used for the assay of amino acids has been determined. Only four were found to require this amino acid: *Lactobacillus casei*  $\epsilon$ , *Lactobacillus pentosus*, *Lactobacillus delbrueckii* LD-3, and *Leuconostoc mesenteroides* PD-60. None of a number of sulfur-containing compounds which were tested could replace cystine in the medium of the last organism mentioned. *L. casei* responds, however, to all compounds tested; glutathione effectively replaces approximately 20 equivalents of cystine in the nutritive medium for this organism.

Sterilization by autoclaving has varying destructive effects upon the activity of cystine and cysteine, depending upon the organism used.

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# THE CHEMISTRY OF THE LIPIDES OF TUBERCLE BACILLI

## LXXV. FURTHER STUDIES ON THE POLYSACCHARIDES OF THE PHOSPHATIDES OBTAINED FROM CELL RESIDUES IN THE PREPARATION OF TUBERCULIN\*

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In an earlier report (1) it was shown that the polysaccharides contained in the phosphatides isolated from cell residues which remained after the preparation of purified protein derivative, the PPD of Seibert, were different from the polysaccharide which is contained in the phosphatide isolated from tubercle bacilli, strain H-37 (2).

Two separate lots of phosphatides were examined at that time and it was found that both yielded different phosphorus-containing polysaccharides or glycosides. There was no explanation possible for such differences, since the various lots of bacilli had been cultivated under identical conditions on the Long synthetic medium (3) and the same strain of bacilli had been used.

It appeared of interest, therefore, to examine the polysaccharide components of other lots of phosphatides prepared from cell residues which remained after the preparation of PPD. Two separate lots of phosphatides were available for this study.

The same strain of tubercle bacilli had been grown under identical conditions on the Long synthetic medium. The preliminary treatment for the preparation of PPD (4) had been the same in every case and the isolation of the phosphatides had been carried out in the same manner as previously employed in this laboratory (5). In view of the uniformity of the bacilli and the processes employed in the preparation of the phosphatides, one would expect that the chemical composition of the polysaccharides isolated from the phosphatides would be identical, but such was not the case.

The polysaccharides isolated from the two lots of phosphatides were dis-

\* The data are taken from the dissertation submitted by G. I. de Sütö-Nagy to the Faculty of the Graduate School, Yale University, 1946, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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† Holder of a National Tuberculosis Association Fellowship in Chemistry at Yale University, 1945-47.

tinctly different and they also differed from the results reported in the previous study (1). The glycoside manninositose (2), which has been found to be a constituent of the phosphatide prepared from tubercle bacilli, strain H-37, was not present.

As a result of these studies on the phosphatides prepared from cell residues it is quite evident that the polysaccharide components differ in composition with every lot of bacilli that were grown. It also appears necessary to conclude that the bacilli were not of the strain H-37.

We have no explanations for the variations that have been observed. They may depend upon mutations or upon other causes as yet unknown. We are merely recording our findings, since they may be helpful to future investigators.

#### EXPERIMENTAL

The phosphatides used in this investigation had been prepared from two separate lots of cell residues designated as Lot 3 and Lot 5. The bacilli designated as strain 2067 had been cultivated in the laboratories of Sharp and Dohme, Glenolden, Pennsylvania, and processed for the production of PPD exactly as described in an earlier paper (1). We are greatly indebted to Sharp and Dohme for providing us with this material. The lipides contained in the cell residues had been extracted and separated in the same manner as was previously employed in this laboratory (5).

The phosphatides had been precipitated ten times from ethereal solution with acetone. The last precipitation had been made by pouring the ethereal solution into ice-cold acetone, which resulted in the formation of nearly white amorphous powders. The phosphatides when dispersed in water formed opalescent solutions which showed an acid reaction to litmus and gave a positive Molisch reaction. The aqueous solutions showed no reduction with Fehling's solution until they had been boiled for several minutes with dilute sulfuric acid.

*The Phosphatide from Lot 3*—When heated in a capillary tube, the phosphatide melted with decomposition at 201–205°. The following values were obtained on analysis.

Found. P 3.78, N 0.67, ash 7.26

*Saponification of the Phosphatide*—The phosphatide, 24.95 gm., was dissolved in 250 cc. of benzene and to the clear solution was added slowly a solution of 6 gm. of potassium hydroxide dissolved in 70 cc. of methyl alcohol. The solution assumed a deep orange color and almost immediately a precipitate began to separate. The mixture was allowed to stand at room temperature with occasional shaking for 20 hours. The precipitate had then collected as a stiff mass on the bottom of the flask. The clear

supernatant solution was decanted and the insoluble material was rinsed out several times with benzene and methyl alcohol and dried *in vacuo*. The substance, which weighed 12.55 gm., was suspended in 175 cc. of alcohol containing 1 per cent of potassium hydroxide and the mixture was refluxed for 2 hours. After the solution had cooled, the insoluble portion was filtered off and washed with absolute alcohol.

The filtrate was freed of excess potassium hydroxide by means of carbon dioxide and the potassium carbonate was filtered off, washed with alcohol, and discarded. The alcoholic filtrate was evaporated to dryness *in vacuo* and the residue was combined with the alcohol-insoluble portion. The total material was dissolved in water, acidified with acetic acid, and the cloudy solution was extracted with petroleum ether. The latter, on evaporation to dryness, left a small residue of fatty acids, which was combined with the main fatty acid fraction contained in the benzene solution from which the fatty acids were isolated and examined, as will be described later.

*Isolation of Phosphoric Acids*—The aqueous solution after extraction with petroleum ether was neutralized with potassium hydroxide and concentrated *in vacuo* to a volume of about 50 cc., after which a solution of neutral lead acetate was added in slight excess, and the lead salts which precipitated were filtered off and washed with water.

The filtrate was reserved for the isolation of carbohydrates.

The lead salts mentioned above were suspended in water and treated with hydrogen sulfide. The lead sulfide was removed and the filtrate was concentrated *in vacuo* to a volume of about 25 cc. The solution was neutralized with barium hydroxide and the inorganic barium phosphate which precipitated was filtered off and discarded. To the filtrate were added 2 volumes of alcohol, which caused a voluminous white precipitate which was collected and washed with alcohol. After the substance had been precipitated three times in the same manner and dried *in vacuo*, it was obtained as a snow-white powder which weighed 4.37 gm. It gave positive Molisch and Scherer reactions, thus indicating the presence of carbohydrate and inositol.

*Isolation of Inositol Monophosphoric Acid*—The barium salt mentioned above was dissolved in water and the barium was removed quantitatively with sulfuric acid. The barium sulfate was filtered off, after which the filtrate was concentrated *in vacuo* to a thick syrup and the latter was triturated with absolute alcohol until a white powder was produced, which was filtered off and washed with absolute alcohol.

The alcoholic solution was examined as will be described below.

The alcohol-insoluble powder mentioned above gave no Molisch reaction but a positive Scherer reaction. The substance was dissolved in



about 20 cc. of water and the strongly acid solution was neutralized with barium hydroxide and the resulting barium salt was precipitated by the addition of 2 volumes of alcohol. The precipitate was filtered off, after which it was reprecipitated three times from aqueous solution with alcohol. The final product after it had been collected, washed with alcohol, and dried *in vacuo* formed a white amorphous powder that was easily soluble in water. For analysis the substance was dried at 107° *in vacuo* over phosphorus pentoxide.

*Analysis*— $C_6H_{11}O_5PBa$  (395.4)

Calculated for barium inositol monophosphate. P 7.84, Ba 34.75

Found. P 7.16, Ba 34.78

The positive Scherer reaction and the composition of the barium salt show that the substance was barium inositol monophosphate.

*Alcohol-Soluble Portion of the Acid*—The alcoholic solution mentioned above on evaporation to dryness *in vacuo* left a thick syrupy residue which gave positive Molisch and Scherer reactions, thus indicating the presence of both carbohydrate and inositol. The substance contained 7.9 per cent of phosphorus and after it had been refluxed for some time with dilute sulfuric acid the solution strongly reduced Fehling's solution. It was evidently a mixture containing a phosphorylated glycoside, but the amount of material available was insufficient for the isolation of any pure substance.

*Examination of Filtrate from Neutral Lead Acetate Precipitate. Isolation of the Carbohydrate*—The filtrate from the neutral lead acetate precipitate, mentioned above, was concentrated *in vacuo* to a volume of about 50 cc. and precipitated by means of an excess of basic lead acetate and ammonium hydroxide. The precipitate was filtered off, washed with 5 per cent ammonium hydroxide, and the excess of ammonia was removed in a vacuum desiccator over sulfuric acid, after which the lead salt was suspended in water and the lead was removed with hydrogen sulfide. The lead sulfide was filtered off and the clear filtrate was concentrated *in vacuo* to a thick syrup.

The syrup was dehydrated by grinding under absolute alcohol until a white powder was produced. The powder was filtered off, washed with alcohol, and dried *in vacuo*. The product which represented the principal carbohydrate fraction weighed 2.77 gm. and gave positive Molisch and Scherer reactions. For analysis it was dried at 78° *in vacuo* over dehydrite.

*Analysis*—Found. P 3.13, N 0.65

Attempts to purify the substance by acetylation will be described later.

*Examination of Alcoholic Filtrate. Isolation of Glycerophosphoric Acid—*

The alcoholic filtrate from the powder mentioned above showed a strongly acid reaction and hence was neutralized with barium hydroxide, whereupon a white precipitate separated which was filtered off, washed with alcohol, and dried *in vacuo*. The substance was treated with 30 cc. of water and the insoluble portion, which consisted of barium phosphate, was filtered off and discarded. To the filtrate were added 2 volumes of alcohol and the resulting white precipitate was collected and washed with alcohol, after which it was reprecipitated in the same manner. After the substance had been dried *in vacuo*, it was obtained as a white amorphous powder that weighed 1.9 gm. It was easily soluble in water and it gave no Molisch or Scherer reaction. For analysis it was dried at 78° *in vacuo* over dehydrite.

*Analysis*—Found. Ba 44.82, 44.86, P 9.23, 9.48

The values found indicate that the substance was barium glycerophosphate.

$C_3H_7O_6P\text{Ba}$  (307.4). Calculated. Ba 44.69, P 10.08

The presence of both phosphoric acid and glycerophosphoric acid in the alcoholic solution must have been due to partial hydrolysis of organic phosphoric acids during the operations involved in the isolation of the carbohydrate.

*Examination of the Carbohydrate*—An attempt was first made to purify a portion of the carbohydrate fraction by acetylation. For this purpose 0.4 gm. of the substance was refluxed with acetic anhydride and fused sodium acetate. The only substance that could be identified, after the reaction mixture had been diluted with water and extracted with chloroform, was a small amount of inositol hexaacetate which was obtained in the form of colorless plate-shaped crystals. It gave the Scherer reaction and melted at 212–213°.

Since the results of the acetylation experiment mentioned above were inconclusive, an attempt was made to fractionate the residue of the material by means of barium salts. The balance of the substance, 1.84 gm., was dissolved in water and the solution, which was acid in reaction, was neutralized with barium hydroxide. A slight precipitate of barium phosphate separated and was filtered off, and 2 volumes of alcohol were added to the clear filtrate. A white amorphous precipitate separated and was collected. The substance, after it had been reprecipitated in the same manner, was obtained as a white powder which weighed 374 mg. and gave positive Molisch and Scherer reactions. It did not reduce Fehling's solution until it had been refluxed for some time with dilute

sulfuric acid. For analysis the substance was dried to constant weight at 78°.

*Analysis*—Found. Ba 28.21, P 7.23

The composition and reactions of this substance indicated that it was a phosphorylated glycoside which contained inositol and carbohydrate, but the small amount of material prevented a complete determination of its constituents.

The filtrate from the above barium salt was freed of traces of barium with sulfuric acid, after which the solution was concentrated *in vacuo* to a thick syrup. It was dissolved in a little water, and alcohol was added to faint cloudiness. On standing, prismatic crystals separated which weighed 0.41 gm. The crystals melted at 222°, and there was no depression when mixed with inactive inositol. The reaction of Scherer was positive; hence the substance was identified as inositol.

The filtrate from the inositol crystals was concentrated *in vacuo* to dryness. The residue was a non-crystallizable syrup which was acetylated in pyridine with acetic anhydride at room temperature. The only crystalline substance that could be isolated from the acetylated material in methyl alcohol solution was a small amount of inositol hexaacetate, m.p. 212°.

After these crystals had been filtered off, the solution was evaporated to dryness and the non-crystalline residue was dissolved in methyl alcohol and saponified with barium hydroxide. After the barium had been removed with sulfuric acid, the solution was refluxed with 5 per cent sulfuric acid for 2.5 hours. The solution was freed quantitatively of sulfuric and phosphoric acids with barium hydroxide. The solution, which gave a strong reduction with Fehling's solution, was concentrated to a volume of 8 cc. and mixed with phenylhydrazine hydrochloride and sodium acetate. A crystalline hydrazone began to separate immediately. After the mixture had stood in the refrigerator overnight, the crystals were filtered off and recrystallized from 60 per cent alcohol. Nearly colorless massive crystals were obtained which weighed 0.14 gm. and melted at 193–195°. There was no depression when mixed with mannose phenylhydrazone. The crystal form and melting point of the hydrazone showed that the reducing sugar was mannose. No other substance could be identified in the reaction mixture.

*Résumé*—The principal water-soluble constituents that were separated from the phosphatide prepared from Lot 3 of the bacterial residues were as follows: inositol monophosphoric acid, glycerophosphoric acid, free inositol, and a phosphorus-containing glycoside which gave Molisch and Scherer reactions. After hydrolysis of this substance the reducing sugar was identified as mannose.

*Examination of the Phosphatide Prepared from Lot 5 of Bacterial Residues after Preparation of PPD*—The phosphatide had been isolated and purified exactly as described for the phosphatide of Lot 3. The properties and composition of the two preparations were practically identical.

*Analysis*—Found. P 3.50, N 0.55, ash 8.16

*Saponification of the Phosphatide*—The saponification was carried out at room temperature as described before. To a solution of 27.1 gm. of the phosphatide was added an excess of potassium hydroxide dissolved in methyl alcohol. A precipitate began to separate almost immediately and after the mixture had stood for 24 hours the supernatant was decanted and the precipitate was collected and washed with benzene and methyl alcohol, after which it was dissolved in water. The resulting alkaline solution was acidified with acetic acid and extracted with ether in order to remove a small amount of fatty acids. The ethereal extract after it had been washed free of acetic acid with water was combined with the benzene solution and the latter was examined as will be described later for fatty acids and glycerol.

*Examination of the Aqueous Solution*—The aqueous solution after extraction with ether was concentrated *in vacuo* to a volume of about 40 cc. and a slight excess of neutral lead acetate was added. The precipitate was collected, washed with water, and decomposed in aqueous suspension with hydrogen sulfide. The lead sulfide was filtered off and the filtrate was concentrated *in vacuo* to a volume of about 25 cc. and neutralized with a solution of barium hydroxide. A precipitate consisting of barium phosphate was filtered off and the filtrate was diluted with 2 volumes of alcohol, which caused a voluminous flocculent precipitate which was collected, washed with alcohol, and dried *in vacuo*. The white powder, 0.77 gm., was dissolved in water and the barium was removed quantitatively with sulfuric acid. The filtrate was concentrated *in vacuo* to a thick syrup and the latter was triturated with absolute alcohol. A small amount of insoluble material was filtered off and examined as will be described later.

The alcoholic solution, which was acid in reaction, was neutralized with barium hydroxide and the resulting precipitate was filtered off and washed with alcohol. The substance was dissolved in water and reprecipitated by adding 2 volumes of alcohol. The white amorphous powder, which weighed 0.57 gm., consisted of barium glycerophosphate as indicated by its composition.

$C_2H_7O_8PBa \cdot \frac{1}{3}H_2O$  (316.4). Calculated. P 9.79, Ba 43.42  
Found. " 9.67. " 43.27

The alcohol-insoluble material mentioned above was dissolved in water and the solution was neutralized with barium hydroxide. The addition of 2 volumes of alcohol gave a white precipitate which was collected and reprecipitated from aqueous solution with alcohol. It was obtained as a white amorphous powder which weighed 0.12 gm. The substance gave no Molisch but a positive Scherer reaction, and when heated with potassium sulfate it gave a strong odor of acrolein.

For analysis the substance was dried *in vacuo* at 110°.

Found. P 9.12, Ba 37.98

A Zeisel determination was made and a volatile iodide was liberated which when calculated as isopropyl iodide corresponded to 7.8 per cent of glycerol. The analytical values suggest that the substance was a slightly impure sample of inositol glycerol diphosphoric acid.

*Isolation of Inositol Glycerol Diphosphoric Acid*—The filtrate from the neutral lead acetate precipitate was freed of lead with hydrogen sulfide and the lead sulfide was filtered off and washed with water. The filtrate was concentrated *in vacuo* to a volume of about 50 cc., after which the acetic acid was removed by extraction with ether. The solution showed a neutral reaction to litmus. It is evident, therefore, that the main portion of the organic phosphoric acids was present as neutral salts which were not precipitated by lead acetate.

It was found, however, that the addition of basic lead acetate to the neutral solution gave a voluminous precipitate which was filtered off and washed with 55 per cent alcohol. The lead salt was suspended in water and decomposed with hydrogen sulfide. The lead sulfide was filtered off and the filtrate was concentrated *in vacuo* to a volume of about 50 cc. The solution, which was strongly acid in reaction, was neutralized with barium hydroxide. A small amount of barium phosphate which separated was filtered off and discarded. The clear filtrate was diluted with 2 volumes of alcohol which caused a voluminous white precipitate which was filtered off and washed with alcohol. The substance was reprecipitated three times from aqueous solution with alcohol. After the precipitate had been dried *in vacuo*, it was obtained as a snow-white powder which weighed 2.313 gm.

The substance, which was easily soluble in water, gave no Molisch reaction, thus indicating the absence of carbohydrate, but it gave a positive Scherer reaction. For analysis the substance was dried *in vacuo* at 110° over phosphorus pentoxide.

*Analysis*—Found. P 9.32, 9.39, Ba 40.05, 39.99

The analytical values agree closely with the calculated composition of a barium salt of the formula  $C_9H_{16}O_{14}P_2Ba_2$  (684.8).

Calculated. P 9.05, Ba 40.12

It will be shown in a separate report that this substance represents a new type of an organic phosphoric acid. On complete hydrolysis the substance yielded 1 part of inositol and 1 part of glycerol together with phosphoric acid. On partial hydrolysis inositol monophosphoric acid and glycerophosphoric acid were obtained.

*Isolation of the Polysaccharide*—The filtrate from the above basic lead acetate precipitate was freed of excess lead with hydrogen sulfide and the lead sulfide was filtered off and washed with water. The filtrate was evaporated *in vacuo* to dryness and the thick syrupy residue was ground in a mortar under absolute alcohol until a white powder was produced. The powder was filtered off, washed with absolute alcohol, and dried *in vacuo*. The substance, which weighed 3.537 gm., was a white amorphous powder which gave positive Molisch, Scherer, and acrolein reactions. An aqueous solution of the substance showed a neutral reaction to litmus and did not reduce Fehling's solution until it had been refluxed for some time with dilute sulfuric acid. The reactions indicated that it contained carbohydrate, inositol, and glycerol.

For analysis the substance was dried at  $110^\circ$  *in vacuo* over phosphorus pentoxide.

Found. P 6.93, ash 27.00, N was absent

The ash in addition to phosphorus contained potassium and magnesium.

Attempts to purify the polysaccharide by precipitation with basic lead acetate and ammonium hydroxide and fractionation by means of barium hydroxide did not lead to any pure or homogeneous compound. A portion of the substance, weight 1 gm., was therefore hydrolyzed by refluxing with 5 per cent sulfuric acid for 3 hours. The amount of reducing sugar liberated corresponded to 32 per cent calculated as glucose.

*Examination of the Cleavage Products*—The solution after hydrolysis contained some phosphoric acid in addition to sulfuric acid, both of which were removed by the addition of barium hydroxide until the reaction was neutral. The insoluble barium salts were filtered off and the filtrate was diluted with 2 volumes of alcohol. The white amorphous precipitate that separated was collected, washed with alcohol, reprecipitated in the same manner, and dried *in vacuo*. It weighed 0.37 gm.

*Analysis*— $C_9H_{16}O_{14}P_2Ba \cdot \frac{1}{2}H_2O$  (316.4). Calculated. P 9.79, Ba 43.42

Found. " 9.80, " 43.81

The composition corresponds to that of barium glycerophosphate.

*Isolation of Mannose As the Phenylhydrazone*—The filtrate from the barium glycerophosphate was freed from a trace of barium with sulfuric acid, after which the solution was concentrated *in vacuo* to a volume of about 12 cc. To the solution was added 0.5 gm. of phenylhydrazine dissolved in 1 cc. of alcohol. Mannose phenylhydrazone began to separate almost immediately and after the mixture had stood overnight the crystals were collected, washed, and dried. The hydrazone, 180 mg., was recrystallized from 60 per cent alcohol and melted at 194–196° with decomposition. There was no depression when mixed with an authentic sample of mannose phenylhydrazone.

*Isolation of Inositol*—The excess of phenylhydrazine in the filtrate from the mannose phenylhydrazone was removed by means of benzaldehyde in the usual manner, and the solution was evaporated to dryness *in vacuo*. From the residue it was possible to isolate after purification about 50 mg. of needle-shaped crystals which gave the Scherer reaction and melted at 220.5°, thus indicating that the substance was inositol.

*Résumé*—The principal water-soluble constituents separated after alkaline saponification of the phosphatide prepared from Lot 5 of the bacterial residues consisted of the following compounds: glycerophosphoric acid, inositol glycerol diphosphoric acid, and a phosphorus-containing glycoside which on hydrolysis gave glycerophosphoric acid, mannose, and inositol.

*Isolation of the Fatty Acids*—The alkaline benzene solution containing the fatty acids was diluted with about 5 cc. of water and refluxed for 2 hours, after which most of the benzene was distilled off. The residue was acidified with dilute hydrochloric acid and extracted with ether. The ethereal extract was washed with water until the washings were neutral to litmus, dried over sodium sulfate, filtered, and evaporated to dryness. The fatty acids thus obtained from the phosphatide of Lot 3 weighed 17.5 gm., equal to 70.14 per cent, and from the phosphatide of Lot 5, 19.26 gm., equal to 71.07 per cent. The two lots of fatty acids were examined as will be described later.

*Isolation of Glycerol*—The aqueous solutions from which the fatty acids had been extracted were examined separately for glycerol. The solutions were neutralized with potassium hydroxide and evaporated to dryness *in vacuo*. The residues were extracted with pyridine and the solvent was evaporated *in vacuo*, leaving thick syrupy residues which consisted of crude glycerol. A portion of the residue in each case was converted into the tribenzoyl derivative and the latter was crystallized from methyl alcohol. Colorless crystals characteristic of glycerol tribenzoate were obtained which melted at 75–76°.

*Examination of the Fatty Acids*—The fatty acids were treated with alcohol and a small amount of insoluble matter was removed by filtration. This material after purification was found to be mycolic acid, m.p. 54–56°.

The alcohol-soluble portion of the acids in as separated into solid and liquid acids by the lead salt-ether procedure. The liquid acids were subjected to catalytic reduction, after which the solid reduced acids were removed by repeating the lead salt-ether treatment several times. Following this preliminary separation the corresponding fractions of the fatty acids from the two phosphatides were combined, esterified by means of diazomethane, and the esters were fractionated by distillation *in vacuo*. The distribution of the fatty acids is shown in Table I.

The solid acids consisted mainly of palmitic acid, but small amounts of higher and lower acids appeared to be present. The solid reduced acids consisted mainly of stearic acid, m.p. 70–71°, molecular weight by titration 284.5, but traces of other acids were present.

The esters of the liquid saturated acids were separated by distillation *in vacuo* into two fractions, a low boiling and a high boiling fraction.

The low boiling ester amounted to 71 per cent of the total esters of the liquid saturated acids and corresponded to methyl tuberculostearate.

TABLE I  
*Fatty Acids of the Phosphatides*

Phosphatide	Total acids	Mycolic acid	Solid acids	Liquid acids	Iodine No. of liquid acids	Reduced solid acids	Liquid saturated acids
gm.	gm.	gm.	gm.	gm.		gm.	gm.
24.95. Lot 3	17.50	0.48	6.10	8.92	32.1	2.70	6.12
27.10. Lot 5	19.25	0.89	6.27	11.65	29.7	2.30	9.15

It was optically inactive,  $n_D^{45} = 1.4368$ . The free acid obtained by saponification of the ester was optically inactive. It was a mobile oil at room temperature, which solidified when cooled in ice water, and melted at 9–12°.

*Titration*—35.45 mg. and 151.08 mg. dissolved in alcohol with phenolphthalein as indicator required 11.95 cc. and 50.96 cc. of 0.01 N alcoholic KOH.

$C_{19}H_{35}O_2$ . Calculated, mol. wt. 298; found, mol. wt. 296, 296

The silver salt was prepared according to Anderson and Chargaff (6) and burned in a porcelain crucible.

$C_{19}H_{37}O_2Ag$  (404.88). Calculated, Ag 26.64; found, Ag 27.14

The properties and composition of the acid indicate that it was tuberculostearic acid.

The higher boiling fraction of the esters of the liquid saturated acids yielded on saponification an acid which was liquid at room temperature



and showed a low dextrorotation. The molecular weight by titration was 372. In ether solution  $[\alpha]_D^{25} = +2.46^\circ$ .

It has been shown in previous reports from this laboratory that the phosphatides prepared from cell residues of tuberculin manufacture yield dextrorotatory, branched chain, liquid saturated fatty acids similar to that isolated in the present investigation. However, no pure phthioic acid has been obtained from these sources. Lack of adequate material has prevented complete purification of these acids which appear to be analogous to phthioic acid.

#### SUMMARY

Two separate lots of phosphatide prepared from cell residues from tuberculin manufacture have been examined. The phosphorus-containing compounds that were liberated on mild alkaline saponification were different.

The phosphatide of Lot 3 gave inositol monophosphoric acid, glycerophosphoric acid, and a glycoside containing phosphorus which gave inositol and mannose.

The phosphatide of Lot 5 gave glycerophosphoric acid, inositol glycerol diphosphoric acid, and a glycoside which on hydrolysis gave glycerophosphoric acid, inositol, and mannose.

Both phosphatides gave about 70 per cent of mixed fatty acids. The liquid saturated fatty acids consisted mainly of tuberculostearic acid, but a small amount of a higher dextrorotatory liquid saturated acid was also present.

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THE CHEMISTRY OF THE LIPIDES OF TUBERCLE BACILLI  
LXXVI. CONCERNING INOSITOL GLYCEROL DIPHOSPHORIC ACID, A  
COMPONENT OF THE PHOSPHATIDE OF HUMAN  
TUBERCLE BACILLI\*

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In studies conducted in this laboratory dealing with the composition of the phosphatide isolated from the human tubercle bacillus, strain H-37, it was recognized early that an organic phosphoric acid of the formula  $C_9H_{20}O_{14}P_2$  could be separated in the form of the barium salt from the water-soluble cleavage products after the phosphatide had been saponified with alcoholic potassium hydroxide (1). This substance was regarded as a complex composed of a hexose monophosphoric acid and glycerophosphoric acid. The acid gave no reduction with Fehling's solution until it had been refluxed for some time with dilute sulfuric acid. The presence of a reducing sugar was thus demonstrated, but the sugar was not identified at that time.

A compound of similar composition was also isolated from the cleavage products of the phosphatide of the leprosy bacillus (2). In a later investigation (3) of the organic phosphorus compounds contained in the phosphatide of the human tubercle bacillus, strain H-37, a barium salt was isolated which corresponded in composition to the formula  $C_9H_{16}O_{14}P_2Ba_2$ . Analysis of this substance indicated that it was mannose glycerol diphosphoric acid. The reducing sugar contained in this compound was identified as mannose.

In addition to the acid of the formula  $C_9H_{20}O_{14}P_2$ , the isolation of glycerophosphoric acid (4), inositol monophosphoric acid (5, 6), and the phosphorus-containing glycoside manninositose (1, 3) has been described.

The isolation of an acid of the formula  $C_9H_{20}O_{14}P_2$  in the form of the barium salt was described in the preceding paper (6) and it was suggested that the substance was inositol glycerol diphosphoric acid. The substance

\* The data are taken from the dissertation submitted by G. I. de Sütö-Nagy to the Faculty of the Graduate School, Yale University, 1946, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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was obtained after alkaline saponification of the phosphatide prepared from Lot 5 of cell residues which remained after the preparation of the purified protein derivative, PPD. Since such a compound has not been isolated or described previously, we present in this paper a brief account of the properties and cleavage products of this unusual acid.

#### EXPERIMENTAL

The barium salt of the acid had been isolated as described in the preceding paper (6) and it was a white amorphous powder which weighed 2.313 gm. It gave no Molisch reaction, but a positive Scherer reaction, and when heated with acid potassium sulfate the evolution of acrolein was evident. In the Zeisel determination a volatile iodide was obtained which when calculated as isopropyl iodide corresponded to 48.3 per cent of barium glycerophosphate.

For analysis the substance was dried over phosphorus pentoxide *in vacuo* at 110°.

$C_9H_{16}O_{14}P_2Ba_2$ (684.8).	Calculated.	P 9.05, Ba 40.12
	Found.	" 9.32, 9.39, Ba 40.05, 39.99

The results of this preliminary examination indicated that the substance contained both inositol and glycerol combined with 2 molecules of phosphoric acid.

*Complete Hydrolysis of the Organic Phosphoric Acid*—In order to determine the cleavage products of the above organic phosphoric acid a portion of the barium salt weighing 1 gm. was heated in a sealed tube with 11 cc. of 10 per cent sulfuric acid for 3 hours and 15 minutes at 160°. There was no pressure on opening the tube and its contents were washed into a beaker. The sulfuric acid and phosphoric acid were removed quantitatively by means of barium hydroxide. The filtrate was evaporated to dryness *in vacuo*, and the residue was triturated with absolute alcohol, which left a white insoluble substance, and the latter was filtered off and washed with alcohol.

*Identification of Inositol*—The alcohol-insoluble substance, after it had been dried *in vacuo* weighed 280 mg. It was dissolved in water, decolorized with norit, and the clear solution was mixed with alcohol to faint turbidity. The solution was warmed until it was clear. On cooling and scratching, colorless needle-shaped crystals separated, which were filtered off after a few hours and twice recrystallized from water by the addition of alcohol. The crystal form was characteristic of inositol and the crystals gave the Scherer reaction. The crystals melted at 222° and there was no depression when mixed with inactive inositol. The properties of the substance identify it as inactive inositol.

*Examination of Alcohol-Soluble Portion of Cleavage Products. Identification of Glycerol*—The alcoholic filtrate and washings were combined and evaporated to dryness *in vacuo*, when a syrupy residue was obtained which weighed 110 mg. The substance, when heated with acid potassium sulfate, gave a strong indication of acrolein, thus suggesting that it was crude glycerol. In order to confirm the presence of glycerol the tribenzoyl derivative was prepared in the usual manner and crystallized from methyl alcohol. The derivative was obtained in the form of colorless needles which melted at 74°, and there was no depression when mixed with an authentic sample of glycerol tribenzoate.

From the results obtained it is evident that the products liberated on complete hydrolysis of the organic phosphoric acid consisted of phosphoric acid, together with inositol and glycerol. By calculation, 1 gm. of a barium salt of the composition  $C_9H_{16}O_{14}P_2Ba_2$  should yield on complete hydrolysis 0.3971 gm. of organic compounds, and if inositol and glycerol were present in equimolecular proportions there should be obtained 0.2628 gm. of inositol and 0.1343 gm. of glycerol. The amounts of inositol and glycerol actually isolated correspond approximately to the theoretical values. The experimental results reported indicate that the organic phosphoric acid was inositol glycerol diphosphoric acid.

*Partial Hydrolysis of the Organic Phosphoric Acid*—The balance of the barium salt was dissolved in water and the barium was removed quantitatively with sulfuric acid. The solution on evaporation to dryness *in vacuo* left a solid residue which was insoluble in alcohol. Attempts to crystallize the acid were unsuccessful.

For partial hydrolysis the acid was dissolved in 10 per cent sulfuric acid and the solution was heated at 45° for about 6 hours. The sulfuric acid was removed with barium hydroxide and the barium sulfate was filtered off. The filtrate was concentrated *in vacuo* to a thick syrup and the latter was triturated with absolute alcohol until a white powder was produced which was filtered off and washed with absolute alcohol.

*Isolation of Barium Inositol Monophosphate*—The alcohol-insoluble substance mentioned above gave a positive Scherer reaction. It was dissolved in a little water and the solution which showed a strong acid reaction was neutralized with barium hydroxide. The clear solution was mixed with 2 volumes of alcohol and the white amorphous precipitate that separated was filtered off and washed with alcohol. After the substance had been reprecipitated from aqueous solution with alcohol, it was collected, washed with alcohol, and dried *in vacuo*. For analysis the substance was dried to constant weight at 78° *in vacuo* over phosphorus pentoxide.

*Analysis*— $C_9H_{11}O_4PBa$  (395.4). Calculated. P 7.84, Ba 34.75  
Found. " 7.38, 7.60, Ba 34.55, 34.14

The properties of the substance and the composition of the barium salt indicate that it was barium inositol monophosphate.

*Isolation of Barium Glycerophosphate*—The alcoholic filtrate from the above alcohol-insoluble inositol monophosphoric acid showed an acid reaction to litmus. The solution was neutralized with barium hydroxide and the precipitate that separated was filtered off and washed with alcohol. The substance, after it had been reprecipitated from aqueous solution by the addition of alcohol, was filtered off, washed with alcohol, and dried *in vacuo*. The product was a snow-white amorphous powder that gave no Scherer reaction. For analysis it was dried at  $110^{\circ}$  *in vacuo* over phosphorus pentoxide.

<i>Analysis</i> — $C_9H_{20}O_{14}P\text{Ba}$ (307.4).	Calculated.	P 10.08, Ba 44.70
	Found.	" 9.93, 10.23, Ba 44.27, 44.62

The analytical values found for the barium salt are in close agreement with the calculated composition of barium glycerophosphate.

#### DISCUSSION

Since it was possible to isolate both inositol monophosphoric acid and glycerophosphoric acid after mild hydrolysis of the acid of the formula  $C_9H_{20}O_{14}P_2$ , it seems evident that the molecules of inositol and glycerol must have been combined either as esters of phosphoric acid or by means of an easily hydrolyzable oxygen bridge. Unfortunately, lack of material prevented a complete investigation of the constitution of the acid, but the results illustrate the diversity of organic phosphoric acids that can be produced by the tubercle bacillus. It would appear also that almost every lot of bacilli that is grown on an artificial medium elaborates different organic phosphoric acid compounds. In future investigations of the organic phosphoric acids isolated from the phosphatide of the human tubercle bacillus it is suggested that special attention should be given to distinguish between mannose glycerol diphosphoric acid and inositol glycerol diphosphoric acid as well as other phosphoric acid compounds. The great variety of metabolic products produced by the organism renders it impossible to predict what compounds may be found.

#### SUMMARY

An organic phosphoric acid of the formula  $C_9H_{20}O_{14}P_2$  was isolated from the phosphatide of a special lot of tubercle bacilli residues from the preparation of tuberculin.

Complete hydrolysis of the acid by heating in a sealed type to  $160^{\circ}$  yielded phosphoric acid and approximately equimolecular quantities of inositol and glycerol.

When the acid was subjected to mild hydrolysis, it yielded inositol monophosphoric acid and glycerophosphoric acid.

It is concluded, therefore, that the acid is inositol glycerol diphosphoric acid, but the chemical constitution of the acid is as yet unknown.

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# THE ULTRAVIOLET ABSORPTION OF SERUM ALBUMIN AND OF ITS CONSTITUENT AMINO ACIDS AS A FUNCTION OF pH

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The absorption of proteins in the ultraviolet in the region of 250 to 300  $m\mu$  is commonly related to the presence in the protein molecule of the aromatic amino acids tyrosine, tryptophan, and phenylalanine (1-6). In general, correlation is excellent between the bands observed in the spectrum of the protein and those of the aromatic amino acids (5, 6). These qualitative relationships have been shown more recently (3, 7, 8) to be quantitative under certain specified conditions with reference to wave-length and pH. Goodwin and Morton (7) have estimated quantitatively tyrosine and tryptophan from the absorption of the protein in 0.1 N NaOH at two different wave-lengths in the ultraviolet. Similarly Lerner and Barnum (8) observed that the absorption of human serum albumin at pH 2 and at 277.5  $m\mu$  corresponded closely to the absorption expected from the chemical analysis of the albumin, which showed the presence of 4.66 per cent tyrosine and 0.19 per cent tryptophan. While this correspondence between the absorption of a protein and of its aromatic amino acids has been shown to hold at a particular wave-length, the correspondence has not been studied quantitatively over the entire ultraviolet spectrum. Neither has the effect of pH on proteins been quantitatively related to its influence on the aromatic amino acids, although Lerner and Barnum (8) noted a shift in the maximum absorption of serum albumin to longer wave-lengths when the pH was changed from 2 to 10. Holiday (3) reported little effect of change in pH upon the absorption of tryptophan or phenylalanine. The absorption peak of tyrosine, however, moved to longer wave-lengths at high pH values, and in addition the absorption below 250  $m\mu$  became greater. Darby (9) has made use of this pH effect to resolve bands due to tyrosine and tryptophan in papain.

The present paper is concerned with correlating quantitatively the ultraviolet absorption of bovine serum albumin with that of the constituent amino acids with special reference to the effect of pH upon this absorption.

## EXPERIMENTAL

The ultraviolet absorption spectra of bovine serum albumin (Armour) and of the amino acids (Eastman) were measured with a Beckman quartz



spectrophotometer. Readings were made at  $2\text{ m}\mu$  intervals except in the region of a maximum or a minimum, when they were made at intervals of  $1\text{ m}\mu$ . The data are recorded as extinction coefficients<sup>1</sup> at each wavelength. Since there was no significant shift in absorption of either the albumin or the amino acids in the acid region as determined in preliminary experiments, subsequent work in acid solution was done only at pH 4.3. The alkaline region is represented by studies made at both pH 10.5 and 12.0.

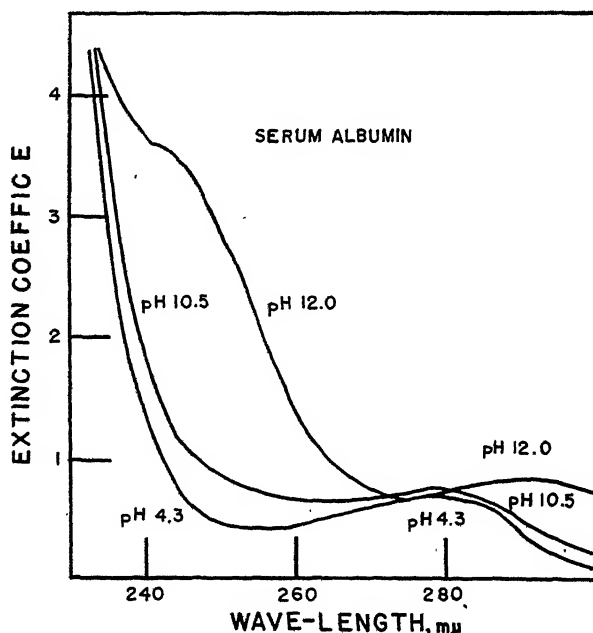


FIG. 1. Absorption in the ultraviolet of crystalline bovine serum albumin (0.333 mg. per ml.) as a function of pH.

In Fig. 1 are shown the absorption curves of serum albumin at pH 4.3, 10.5, and 12.0 (pH adjusted to the appropriate value with dilute NaOH or dilute HCl). The shift to higher wave-lengths of both the maximum and minimum absorption with pH is brought out more clearly in Fig. 2. The characteristics of the absorption in the ultraviolet (Fig. 1) of crystalline serum albumin are not caused by low molecular weight impurities which might be present, for when these are removed by dialysis the absorption

<sup>1</sup> The extinction coefficient is obtained from the relationship, optical density =  $\log I_0/I_x = kcx$ , where  $I_0$  and  $I_x$  are the incident and transmitted light respectively,  $x$  the cuvette diameter in cm.,  $c$  the concentration in gm. per liter, and  $k$  is the extinction coefficient.

curve is unaltered. Lerner and Barnum (8) observed a similar shift when they changed the pH of human serum albumin from 2 to 10. More striking than the effect of pH upon the wave-length of maximum and minimum absorption, however, is the plateau in the curve for pH 12, which appears at about  $240\text{ m}\mu$  (Fig. 1). This effect is not evident in the work of Lerner and Barnum (8), since they did not explore the region above pH 10.

An attempt to interpret the data on the ultraviolet absorption of serum albumin was made by studying the ultraviolet absorption of the constituent amino acids of serum albumin. A synthetic mixture was prepared which

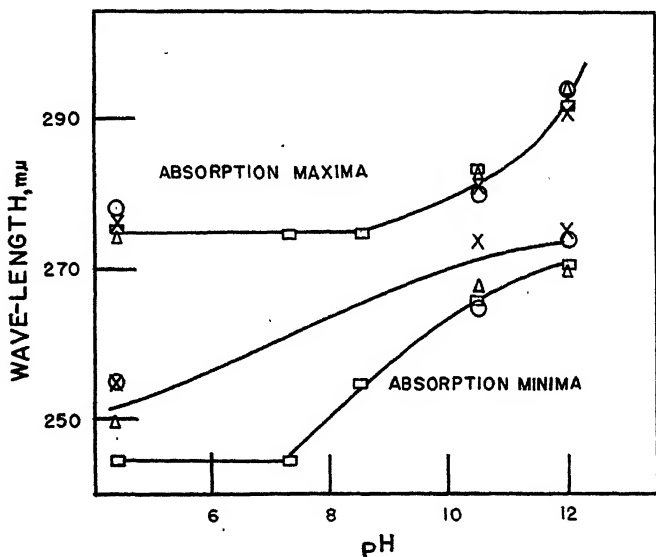


Fig. 2. Ultraviolet absorption maximum and minimum as a function of the pH of the medium. O, bovine serum albumin (data from Fig. 1); Δ, amino acid mixture (data from Fig. 3); □, L-tyrosine (data from Fig. 4); X, peptic digest of bovine serum albumin (data from Fig. 5).

contained the amino acids in the same proportions as they exist in the protein (10). The ultraviolet absorption as a function of pH is presented in Figs. 2 and 3. The wave-lengths of the maximum and minimum and their shift with pH are strikingly similar to those for serum albumin. Although at pH 4.3 the absorption in the ultraviolet of the amino acid mixture is practically identical in every respect with that of serum albumin (compare Figs. 1, 2, and 3), marked differences occur in the region of  $240\text{ m}\mu$  at pH 10.5 and 12.0. A plateau characterizes the amino acid mixture, but not the albumin, at pH 10.5, while at pH 12.0 there is a sharp peak in absorption for the mixture, but only a plateau in the albumin curve at  $240\text{ m}\mu$ .

In attempts at interpretation of the ultraviolet curves of the amino acid mixture, detailed studies were made of the absorption in the ultraviolet of the aromatic amino acids, phenylalanine, tryptophan, and tyrosine. The results with the first two are summarized in Table I, from which it is apparent that only a slight shift in absorption spectrum occurs as a function of pH. With neither phenylalanine nor tryptophan is there any evidence in strongly alkaline solutions of a plateau or peak in the absorption curve at 240  $m\mu$ . For tyrosine, however, the situation is very different (Fig. 4);

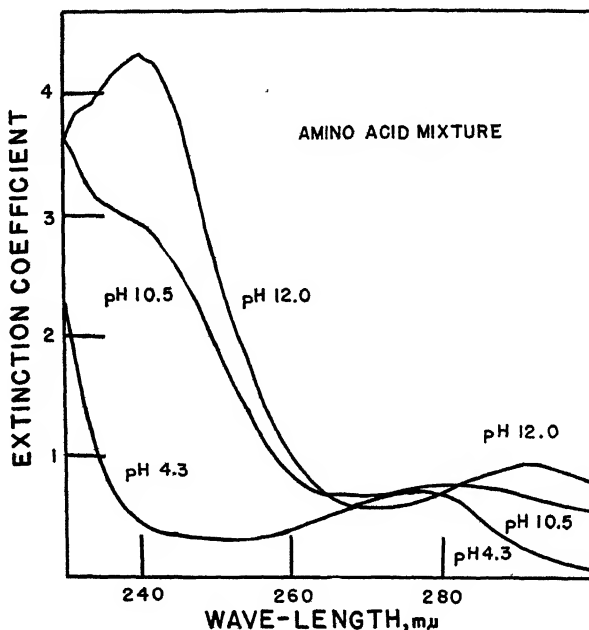


FIG. 3. Absorption in the ultraviolet as a function of pH of amino acids (0.333 mg. per ml.) mixed in the proportions in which they exist in serum albumin.

both the usual maximum and minimum shift to higher wave-lengths as the pH is increased in the alkaline region. This shift, as shown in Fig. 2, is closely analogous to that for serum albumin. The wave-length of the maximum for serum albumin as well as the shift in maximum with pH is accounted for to a high degree of precision by the tyrosine present in the protein. The minimum and its shift with pH are not quite the same for tyrosine and serum albumin except in strongly alkaline solutions. At lower pH values it is clear that other amino acids contribute in determining the wave-length of minimum absorption of serum albumin.

The plateau in the absorption curve at 240  $m\mu$  of serum albumin at pH

12.0 can be explained on the basis of the presence in the protein of tyrosine, which shows at 240  $m\mu$  a plateau at pH 10.5 and a sharp peak at pH 12.0. The shift of the 245  $m\mu$  minimum and of the 275  $m\mu$  maximum of tyrosine to longer wave-lengths with increase in alkalinity has been observed by

TABLE I  
*Absorption Maxima and Minima in Ultraviolet of DL-Phenylalanine and of DL-Tryptophan As Function of pH*

pH	Phenylalanine		Tryptophan	
	Minimum	Maximum	Minimum	Maximum
	$m\mu$	$m\mu$	$m\mu$	$m\mu$
4.3	230	258	243	278
10.5	234	259	244	281
12.0	236	259	245	281

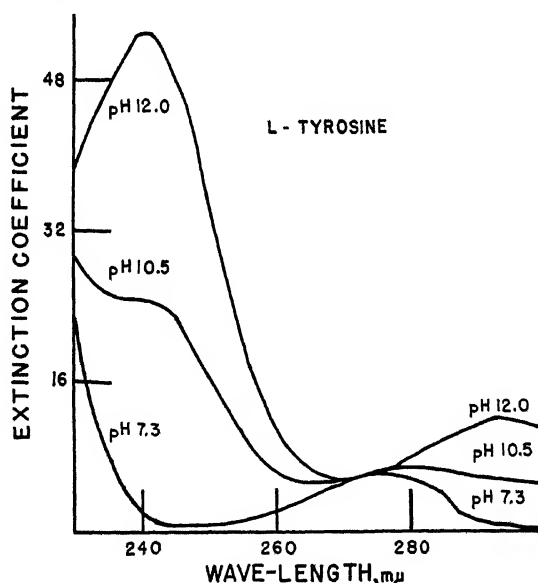


FIG. 4. Absorption in the ultraviolet of L-tyrosine (1.0 mg. per ml.) as a function of pH.

others (3, 8) who ascribed this shift to ionization in alkali of the phenolic hydroxyl group of tyrosine. The maximum at 240  $m\mu$  in strongly alkaline solutions reported here has not been observed before, but the suggestion is made that it is also related to the formation of the phenoxide group. Greenberg and Barnum (11) have recently studied the dissociation of the

phenolic hydroxide group of tyrosine and report a  $pK$  of approximately 10. Since the absorption of the dissociated and undissociated groups is mutually independent (12), the absorption of an equal mixture of the phenoxide ion and of tyrosine with the undissociated phenolic group will represent the sum of the absorption of the two. At pH 12.0 the ionization should be complete and hence the peak at  $240\text{ m}\mu$  characteristic of the phenoxide ion becomes apparent. The extinction coefficient of this maximum at pH 12.0 is 53 (see Fig. 4). At pH 7.3, however, the phenolic group is almost completely undissociated, and the corresponding extinction coefficient is 2.0. At pH 10.5 the concentration of the phenoxide ion and of the tyrosine with an undissociated hydroxyl group should be about equal, and the extinction coefficient at  $240\text{ m}\mu$  should therefore be the average of the two, or 27.5. The good agreement of the observed coefficient for pH 10.5 of 24.6 at  $240\text{ m}\mu$  with the theoretical value is evidence in favor of the concept that ionization of the phenolic group accounts for the change in absorption of tyrosine as a function of pH.

It appears that in serum albumin as in tyrosine the shift in absorption with pH is related to ionization of the phenolic group. Failure of the serum albumin to show the pronounced absorption at pH 10.5 at  $240\text{ m}\mu$ , typical of tyrosine, must mean that in serum albumin the phenolic groups are bound in some way that prevents ionization. This binding must be of a chemical type and not due to a mutual interaction of the constituent amino acids, since in the amino acid mixture (Fig. 3) the phenolic groups are free to ionize. Studies made with the amino acid mixture (Merck) from which the tyrosine had been omitted clearly indicate that almost all of the characteristic absorption in the ultraviolet of serum albumin and of its constituent amino acids can be accounted for by tyrosine.

Information was sought on the binding of phenolic groups in serum albumin by studying the effects of hydrolysis upon the absorption in the ultraviolet, since it seemed likely that hydrolysis might sufficiently alter the configuration of the protein molecule so as to permit the ionization of phenolic groups. For enzymatic hydrolysis, pepsin is one of the most interesting enzymes to use in view of its ability to cleave peptide bonds involving tyrosine or phenylalanine (13, 14). 100 mg. of serum albumin were digested for 2 hours at pH 2.0, at  $37^\circ$  with 1.0 mg. of crystalline pepsin (Lehn and Fink). Hydrolysis by pepsin<sup>2</sup> was extensive, as is indicated by the failure of any precipitate to form when trichloroacetic acid was added to the digest, and by the fact that after dialysis of the digest the residue contained no tyrosine as measured by ultraviolet absorption or by chemical analysis (15). The effect of pH on the ultraviolet absorption of the peptic digest of serum albumin is shown in Fig. 5. As might be ex-

<sup>2</sup> Suitable controls showed that hydrolysis was not caused by the acid present.

pected, the results are somewhat intermediary between those for serum albumin (Fig. 1) and for the synthetic mixture of amino acids (Fig. 3). Peptic digestion has caused a small peak at  $240\text{ m}\mu$  to occur at pH 12.0, and a definite change in slope at  $240\text{ m}\mu$  to appear at pH 10.5. The addition of 1.0 mg. of trypsin (Pfanstiehl) to the peptic digest and adjustment of the pH to 8.5 result in a much more extensive splitting of peptide bonds;

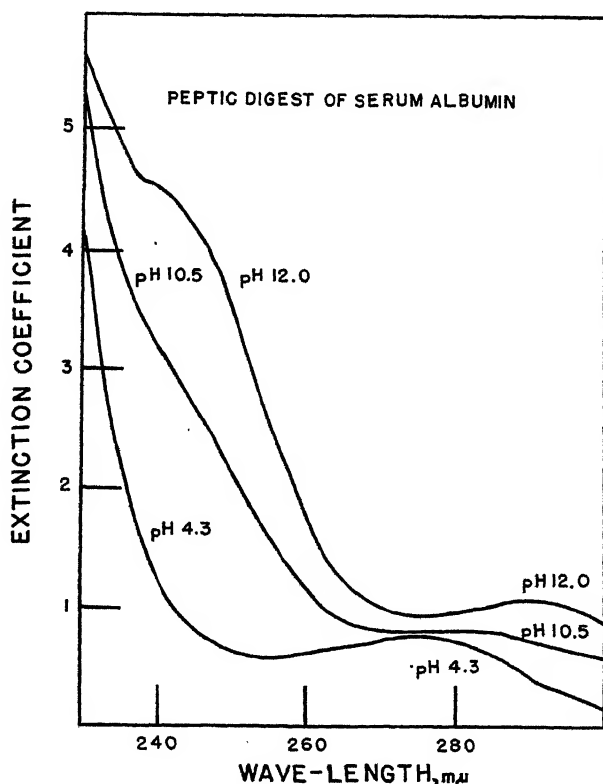


Fig. 5. Absorption in the ultraviolet as a function of pH of a peptic digest of serum albumin (0.333 mg. per ml.).

so that the resulting curves (at different pH values) for absorption in the ultraviolet resemble those for the amino acid mixture more closely than those for the native serum albumin.

If trypsin is used as the digestive agent (1 mg. of trypsin added to 100 mg. of albumin at pH 8.5 for 18 hours at  $37^\circ$ ), the digestion is only about two-thirds as extensive as with pepsin (when measured by tyrosine determination on the undigested protein). Similarly the resulting curves for absorption in the ultraviolet, while lying between the two, resemble some-

what more closely the original protein than they do the mixture of amino acids. These results are noteworthy in view of the fact that, unlike pepsin, trypsin requires the presence of basic amino acids rather than tyrosine or phenylalanine in order to attack a peptide bond (13).

Since alkaline hydrolysis is frequently used as a preliminary step in the analysis of many amino acids, it was also employed in this study to determine its effect on the absorption in the ultraviolet. Alkaline hydrolysis was performed in the usual manner (16) by boiling the serum albumin for 18 hours in 6 N NaOH. The absorption curves of the hydrolyzed protein

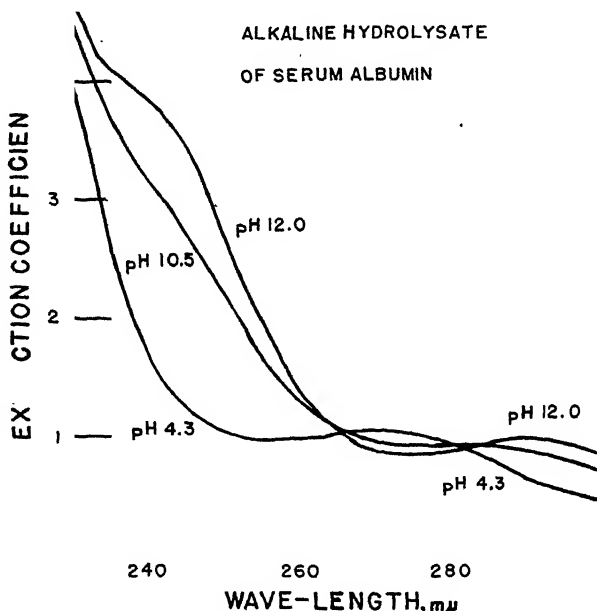


Fig. 6. Absorption in the ultraviolet as a function of pH of an alkaline hydrolysate of serum albumin (0.333 mg. per ml.).

(Fig. 6) clearly show, from the absorption at 240 mμ, the presence of the phenoxide ion at pH 10.5 and 12.0, but its presence is not nearly as evident as in the amino acid mixture. This is surprising, since it is generally accepted that digestion with boiling alkali brings about complete hydrolysis. Other effects of treatment with alkali appear when the maxima and minima are examined. At pH 4.3 the usual minimum at 255 mμ has been almost eliminated and the maximum at 270 mμ has only a slightly higher extinction coefficient than the minimum. A similar situation prevails at pH 10.5, and, to a much lesser extent, at pH 12.0. The failure of alkaline treatment to bring out the expected peak in absorption at 240 mμ at pH 12.0, together

with the effects of alkali on the relative extinction coefficients at 255 and 270  $m\mu$ , indicates that hot alkali has modified the absorption in the ultraviolet of tyrosine and probably of other amino acids.

#### DISCUSSION

The absorption in the ultraviolet of serum albumin can be interpreted in terms of the absorption of the tyrosine present in the molecule. The correlation is excellent at the maximum of 278  $m\mu$  at all pH values studied, but for the minimum at about 260  $m\mu$  this correlation is good only in strongly alkaline solutions (Fig. 2). In less alkaline solutions the amino acids tryptophan and phenylalanine as well as tyrosine contribute toward determining the extinction coefficient of the minimum. The change in shape of the curve at 240  $m\mu$  of the serum albumin at pH 12.0 seems to be determined by the dissociation of the phenolic group of tyrosine. Failure to find evidence of the phenoxide ion at pH 10.5 from the absorption of serum albumin at 240  $m\mu$  indicates that in the albumin molecule this group must be combined. The bond can hardly be a very stable one, however, for at pH 12.0 it is partly broken, allowing some of the phenolic groups to ionize. A hydrogen bond seems to be the most plausible type of linkage to be involved. A hydrogen bond might form between the amino group of lysine, which extends 7.54 Å from the backbone of the polypeptide chain, and the phenolic group of tyrosine, which is also about 7.3 Å from the backbone (calculated from Pauling (17)). Basic amino acids other than lysine, however, might also be involved in forming this type of bond.

Since the plateau in the curve at 240  $m\mu$  of serum albumin at pH 12.0 is ascribed to the ionization of phenolic groups, it might be expected that a similar plateau (or even a peak) would occur in the curve for other proteins. Crystalline insulin (Armour) (final concentration 0.0667 mg. per ml.) at pH 12.0 shows a definite peak in absorption at 241  $m\mu$  in addition to the usual maximum at 293  $m\mu$ . In their study of insulin, Crammer and Neuberger (18) attributed the shift with pH of the absorption maximum at 280  $m\mu$  to ionization of the phenolic group of tyrosine. It is interesting to note that insulin contains a much higher tyrosine content than does serum albumin and that its tyrosyl groups are more reactive with tyrosinase than are those of serum albumin (19). Experiments are in progress to relate the absorption at 240  $m\mu$  at pH 12.0 of other proteins to their tyrosine content and reactivity of the tyrosyl groups.

The results of enzymatic hydrolysis in general confirm earlier data (see (8)) that proteases have practically no effect on the absorption of proteins in the ultraviolet. As a result of cleavage of peptide bonds by the enzyme, some of the bonds (hydrogen ?) attached to phenolic groups become broken, so that after digestion the phenoxide ions (as indicated by absorption at



240  $m\mu$ ) are more apparent in strongly alkaline solutions. On the other hand, hydrolysis by boiling in alkali has much more drastic effects in altering the absorption of serum albumin in the ultraviolet. Since phenolic groups are also released by alkali, the absorption at 240  $m\mu$  in strongly alkaline solutions is also increased. In view of the effects of pH and of alkali upon the absorption in the ultraviolet, any quantitative methods for determination of the aromatic amino acids based upon their absorption in the ultraviolet will require careful attention to experimental detail and caution in interpretation.

The authors wish to acknowledge the assistance in this work rendered by Miss Janette Robinson and by Mrs. Gloria Peacock.

#### SUMMARY

The maximum and minimum absorption of tyrosine moves progressively to longer wave-lengths in the ultraviolet as the pH is increased above pH 7.0. In solutions of pH 12.0 a second sharp absorption maximum appears at 240  $m\mu$ , which is ascribed to the ionization of the phenolic group of tyrosine. The ultraviolet spectra of tryptophan shifted only slightly with pH, while those of phenylalanine are relatively independent of pH.

The ultraviolet absorption spectrum, and the effect of pH thereon, of bovine serum albumin can be interpreted almost quantitatively on the basis solely of the tyrosine present in the protein. The major difference lies at 240  $m\mu$  at pH 12.0, where serum albumin shows only a plateau in the absorption curve as compared with the peak for tyrosine. The difference is interpreted as indicating that in the protein most of the phenolic groups are bound, probably by hydrogen bonds, and are not free to ionize. When the protein is hydrolyzed by pepsin, trypsin, or alkali, the phenolic groups are set free to ionize, as is indicated by an increase in absorption caused by the phenoxide ion at 240  $m\mu$  in strong alkali. Although the action of proteases has little additional effect on the over-all absorption, alkaline hydrolysis modifies the shape of the absorption curve, making it in general unsuitable for quantitative analysis of the constituent amino acids.

An absorption maximum at 241  $m\mu$  at pH 12.0 is also apparent in the absorption curve of insulin as well as of serum albumin and can probably be related to the concentration of phenoxide ions in most proteins.

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# THE ACTION OF CHOLINE AND FAT ON LIPIDE PHOSPHORYLATION IN THE LIVER\*

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Earlier studies with the aid of radioactive phosphorus as an indicator have shown that the rate of the formation of phospholipides in the liver of rats was definitely higher when the diet contained large proportions of fats (1). It seems probable that in these experiments adequate amounts of choline or choline precursors were available to the animals. On the other hand the administration of choline to rats on low protein diets markedly stimulates the turnover of liver phospholipides as measured by the introduction into their molecule of either radioactive phosphorus (2-4) or isotopic choline nitrogen (5). In these studies only high fat diets were employed.

In the present investigation an attempt has been made to study, under strictly comparable conditions, the action of single large doses of choline or fat, or both, on the formation of phospholipides in the liver of rats previously maintained on a low fat, low protein diet. For the purpose of comparison a few experiments were made on rats maintained on an adequate stock diet. Moreover, in most of our animals, determinations were also carried out on the lipides of the small intestine. It was hoped that the results of these determinations could supply additional information on the lipide phosphorylation in the intestine after administration of choline and fat. Preliminary findings on this subject have already been described and discussed briefly (6).

## EXPERIMENTAL

Male albino rats raised on a stock diet<sup>1</sup> to 100 to 110 gm. of body weight were maintained for 7 days on an experimental diet (Diet 26) containing casein 5 parts, dextrin 42, sucrose 42, Crisco 4.0, cod liver oil 1.0, salt mixture (7) 4, Ruffex 2.0. A solution of pure B vitamins, in the amounts previously indicated (8), was incorporated in the daily ration of the animals. During the week on the experimental diet, the weights of the rats remained unchanged, or, more often, decreased slightly.

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<sup>1</sup> Rockland Farms diet for rats, complete. This diet contains approximately 25 per cent protein and 5 per cent fat.

In each experiment, four rats, A, B, C, and D, were employed, to which the following were given by stomach tube: Rat A, water (1 cc.); Rat B, choline chloride (30 mg. in 1 cc. of water); Rat C, oil (2.2 cc.)<sup>2</sup> and 5 minutes later choline chloride (30 mg. in 1 cc. of water); Rat D, oil (2.2 cc.) and 5 minutes later water (1 cc.). 5 minutes after the last administration, the rats were injected intraperitoneally with a solution of  $\text{Na}_2\text{HPO}_4$  containing radioactive phosphorus.<sup>3</sup> The volume, the phosphorus content, and the radioactivity of the solution injected were identical in each experiment, but varied in different experiments between 0.5 and 1 cc., 0.1 and 0.2 mg. of P, and 1 and 3 microcuries, respectively. These variations are slight enough not to interfere appreciably with a comparison between the results obtained in the various experiments (9).

After a certain time interval from the introduction of radioactive phosphorus, the four animals were killed by decapitation. The liver and, usually, the small intestine were removed, rapidly weighed, and minced under alcohol. The lipides were then extracted with hot alcohol, alcohol-ether, and chloroform. On aliquots of the chloroform solution, the weight of the lipide extract, the phosphorus content, and the radioactivity were determined as previously described (10), except that for the radioactive measurements the chloroform solution, after being shaken with sodium phosphate, was centrifuged in glass-stoppered tubes and non-radioactive lipides from rat livers were used in bringing to a definite value the weight of the dry materials in both unknowns and standards. The results of these measurements have been expressed in relative radioactivity units (r.r.u.), the dose injected into the animal being considered equal to  $10^4$  r.r.u. The number of r.r.u. in the whole liver or small intestine has been indicated as the "total radioactivity." In the calculation of the specific activity, in order to compensate for the slight differences in the size of the animals, the values for the lipide P were referred to the whole liver or the whole intestine of a rat with a terminal body weight of 100 gm., and the specific activity obtained by dividing the total radioactivity (in r.r.u.) by the corrected values of lipide P (in mg.).

In several experiments the choline content of the chloroform extracts was also determined. Thus, approximate values for the total phospholipides (mg. of P  $\times$  22.7), the choline-containing phospholipides (mg. of

<sup>2</sup> A commercial preparation of partially hydrogenated cottonseed oil was given. Since notable portions of the oil remained in the syringe and in the stomach tube, in several experiments the amounts of oil actually ingested by the animals were determined; the values obtained by differential weighing averaged approximately 1.5 gm.

<sup>3</sup> The  $\text{P}^{32}$  used in these experiments was supplied by the Physics Department of the Massachusetts Institute of Technology, Cambridge, Massachusetts, and by the Clinton Laboratories, Oak Ridge, Tennessee, on allocation from the United States Atomic Energy Commission.

choline  $\times 6.7$ ), and the total lipides (weight in mg. of the chloroform extract) could be estimated (11). In two experiments, a sample of the liver was extracted with trichloroacetic acid, the inorganic phosphate was precipitated with the strychnomolybdic reagent, and the phosphorus content and radioactivity were determined in the precipitate (10). Within the limits of error of the determinations, the specific activity of the inorganic phosphorus was the same in the liver of Rats A, B, C, and D.

### Results

*Determinations on Liver of Rats Killed at Various Intervals after Injection of  $P^{32}$* —In these orienting experiments, the animals were killed 3, 6, 12, and 24 hours after the administration of the isotopic phosphate. From the results (which are not reported for the sake of brevity), it appears that, for each series of Rats A, B, C, and D, the specific activity-time curves of the lipide phosphorus in the liver exhibit the same general aspect. In all four conditions, the values increase progressively during the first 6 hours of the experiments, and become lower in the longer periods. It might be mentioned that in the experiments of Perlman and Chaikoff (2) the specific activities reached their peak about 8 hours after administration of the radioactive phosphorus.

*Determinations on Liver of Rats Killed at 6 Hour Interval after Injection of  $P^{32}$* —In Table I the individual data obtained in the experiments made at this time interval are recorded. It is apparent that in the animals on Diet 26 the total radioactivity values are increased after choline administration, and even more markedly when oil and choline were given. In most of these experiments, the analytical values for the lipide phosphorus exhibit changes in the same direction. However, these changes are proportionately smaller than the corresponding changes in the total radioactivity; consequently the specific activity values are also usually higher in Rats B than in Rats A, and highest in Rats C.

For both radioactivity and lipide phosphorus the values obtained after administration of oil and water are essentially the same as those found in the liver of rats receiving water only.<sup>4</sup>

<sup>4</sup> Detailed values for total lipides and choline-containing phospholipides are not reported for the sake of brevity. In five experiments the average figures for the whole liver of 100 gm. rats maintained on Diet 26 and killed at the 6 hour interval were as follows: Rats A, B, C, and D, total lipides, mg., 319, 281, 412, and 505, choline-containing phospholipides, mg., 40, 54, 63, and 42, choline-containing phospholipides, per cent of total phospholipides, 46, 56, 61, and 47, respectively. These values may perhaps be suggestive (for instance, for a lipotropic effect of choline even within the short time after its administration, and for changes in the amounts of lecithins proportionately greater than those in the total phospholipides), but, in view of their small magnitude and the extent of the individual variations, none of these differences is significant.

As for the experiments on the rats on the stock diet, most of the figures for the total radioactivity were within the same range as those found in the liver of the corresponding Rats A maintained on the experimental diet. Since, in agreement with previous observations (12), the livers of the rats on the stock diet often had a higher phospholipide content, the specific

TABLE I  
*Lipide Phosphorylation at 6 Hour Interval in Liver of Rats on Low Fat, Low Choline Diet (Diet 26) and on Stock Diet*

Experiment No.	Total radioactivity in lipides				Lipide phosphorus (in liver of 100 gm. rat)				Specific activity of lipide phosphorus			
	Rat A. H <sub>2</sub> O	Rat B. Cho- line	Rat C. Oil + cho- line	Rat D. Oil + H <sub>2</sub> O	Rat A. H <sub>2</sub> O	Rat B. Cho- line	Rat C. Oil + cho- line	Rat D. Oil + H <sub>2</sub> O	Rat A. H <sub>2</sub> O	Rat B. Cho- line	Rat C. Oil + cho- line	Rat D. Oil + H <sub>2</sub> O
Diet 26												
1	331	514*	653*	363	3.41	5.04	5.53	3.95	97	102*	118*	92
2	456	495†	495†	385	4.60	4.90	4.63	4.53	99	101†	107†	85
3	446	512	690	449	3.88	3.51	5.15	3.97	115	146	134	113
4	382	406	616	385	4.06	4.06	4.34	4.14	94	100	142	93
5	307	394	586	281	3.57	3.83	4.41	3.02	86	103	133	86
6	362	548	669	371	3.15	3.86	4.21	4.08	115	142	159	91
7	325	493	515	325	3.65	3.97	4.33	3.61	89	124	119	90
Average..	373	480	603	366	3.76	4.17	4.66	3.90	99	117	130	93
Stock diet												
8†	344	382	548		4.35	4.20	4.94		79	91	111	
			483				3.86				125	
			652				6.04				108	
9	516	430	481	457	5.73	5.66	6.50	5.13	90	76	74	89
10	342	437	372	390	4.79	5.68	5.10	5.57	67	77	73	70
11	335	359	387	378	5.00	5.90	5.69	5.64	67	61	68	67
Average..	384	402	487	408	4.97	5.36	5.36	5.45	76	76	93	75

\* These rats received 100 mg. of choline chloride each.

† These rats received 45 mg. of choline chloride each.

‡ The five rats employed in this experiment weighed between 200 and 250 gm.

activity values were usually lower than in the corresponding rats on Diet 26. In one experiment (No. 8, in which older rats were employed), the effects of the administration of choline only, or choline and oil, were similar to those described for the rats on the experimental diet. However, in the other three experiments, no marked or consistent changes were noted after giving choline, or choline and oil, or oil only.

*Determinations on Small Intestine of Rats Killed at 6 Hour Interval after Injection of  $P^{32}$* —On the basis of previous results (6), the specific activity values for the lipides of the small intestine reach their peak 6 hours after the injection of  $P^{32}$ . The additional data obtained in the present experiments at this time interval are recorded in Table II. As in the liver, in the intestine of rats on Diet 26 the total radioactivity values were higher

TABLE II  
*Lipide Phosphorylation at 6 Hour Interval in Small Intestine of Rats on Low Fat, Low Choline Diet (Diet 26) and on Stock Diet*

Experiment No.	Total radioactivity in lipides				Lipide phosphorus (in small intestine of 100 gm. rat)				Specific activity of lipide phosphorus			
	Rat A. $H_2O$	Rat B. Choline	Rat C. Oil + choline	Rat D. Oil + $H_2O$	Rat A. $H_2O$	Rat B. Choline	Rat C. Oil + choline	Rat D. Oil + $H_2O$	Rat A. $H_2O$	Rat B. Choline	Rat C. Oil + choline	Rat D. Oil + $H_2O$
Diet 26												
2	123	162*	181*	193	2.67	3.31	3.12	3.27	46	49*	58*	59
3	149	169	226	140	2.37	2.11	2.83	2.15	63	80	80	65
4	125	116	308	122	2.36	1.97	3.11	2.35	53	59	99	52
5	120	184	229	125	2.31	3.07	3.42	2.78	52	60	67	45
6	120	183	209	168	2.14	2.32	2.43	3.05	56	79	86	55
7	88	171	204	133	2.05	2.11	3.23	2.77	43	62	63	48
Average..	121	164	226	147	2.32	2.48	3.02	2.73	52	65	76	54
Stock diet												
9	117	145	233	187	2.25	2.79	3.76	2.79	52	52	62	67
10	121	123	177	161	2.37	2.67	2.95	2.98	51	46	60	54
11	100	135	163	127	2.17	2.60	3.13	2.36	46	52	52	58
Average..	113	134	191	158	2.26	2.69	3.28	2.71	50	50	58	60

\* These rats received 45 mg. of choline chloride each.

in Rats B, receiving choline, than in Rats A, to which no choline was given, and highest in Rats C, which received both choline and oil.

Similar increases were sometimes apparent in the lipide phosphorus figures. Since, however, these increases were relatively less considerable than the increases in the isotopic values, the specific activity values were greater in Rats B than in Rats A, and, in four out of six experiments, also greater in Rats C than in Rats B. In several experiments the administration of oil, even without choline, appeared to cause a quite definite increase in both the radioactivity and the analytical values of the lipide phosphorus,



and, at least in two experiments (Nos. 2 and 7), the specific activity was also distinctly higher in Rats D than in Rats A.<sup>5</sup>

In the experiments on the rats maintained on the stock diet, the effects of choline without oil are less marked and approximately of the same magnitude for the radioactivity and the lipide phosphorus values. On the other hand, all figures, including those for the specific activity, were generally

TABLE III

*Comparison of Effects of Choline or Choline and Fat on Lipide Phosphorylation in Rat Liver and Small Intestine\**

All rats were maintained on a low fat, low choline diet for 7 days and killed 6 hours after injection of P<sup>32</sup>.

		$\bar{B} - \bar{A}$	$\bar{C} - \bar{D}$	$\bar{C} - \bar{B}$	$\frac{(\bar{C} - \bar{D}) - (\bar{B} - \bar{A})}{2}$
Liver ( $n = 12$ )					
Total radio-activity	Difference between means	107.6	237.8	123.1	130.2
	$t$	3.46	8.06	3.42	4.24
Specific activity	Difference between means	17.6	37.4	13.5	19.8
	$t$	1.98	4.07	1.34	2.35
Intestine ( $n = 10$ )					
Total radio-activity	Difference between means	43.4	79.4	62.0	36.0
	$t$	3.35	3.27	3.02	1.19
Specific activity	Difference between means	12.6	21.5	10.7	8.9
	$t$	2.18	3.06	1.32	1.18

\*  $\bar{A}$ ,  $\bar{B}$ ,  $\bar{C}$ ,  $\bar{D}$  are the means of the values for the total radioactivity (in r.r.u.) and the specific activity obtained in rats receiving water, choline, choline and oil, and water and oil, respectively;  $t$  is the test of significance as applied to the difference between means;  $n$  is the degree of freedom. For  $n = 12$  and  $n = 10$  the values of  $t$  indicating a probability of a chance occurrence of 5 in 100 are 2.179 and 2.228, respectively. For a probability of 1 in 100, the corresponding values of  $t$  are 3.055 and 3.169.

higher in the intestine after the simultaneous administration of oil and choline, and also when oil and water but no choline were given.

*Statistical Evaluation of Results of Determinations on Rats on Diet 26 Killed at 6 Hour Interval after Introduction of P<sup>32</sup>*—The number of the data

<sup>5</sup> It should be pointed out that our determinations were carried out on the whole intestine. Therefore, our values include the lipides of the mucosa as well as those of the muscle. In the latter the formation of phospholipides occurs at a slower rate (1). If the radioactivity and the phosphorus had been estimated in the lipides of the separated mucosa, one may well expect that the specific activities would have been much higher. Presumably also more marked differences between these values would have been found.

available is perhaps too small to allow quite definite conclusions. However, an attempt has been made to determine whether the average values for the total radioactivity and the specific activity, obtained in each series of Rats A, B, C, and D, are significantly different. The differences between the means and the values of  $t$  (13) corresponding to these differences are reported in Table III. For the total radioactivity, the differences expressing the action of choline without oil ( $\bar{B} - \bar{A}$ ) or with oil ( $\bar{C} - \bar{D}$ ) were found to be highly significant in both liver and intestine, with a probability of chance occurrence ( $P$ ) less than 1 in 100. The specific activities were significantly different only when choline was given with the oil.

The enhancement of the action of choline by the simultaneous administration of oil might be indicated, either (1) by the difference between the average values obtained in Rats C (receiving oil and choline) and Rats B (receiving choline alone), or (2) by the difference between the differences ( $\bar{C} - \bar{D}$ ) and ( $\bar{B} - \bar{A}$ ). The significance of the values obtained with either method is high for the total radioactivities in the liver ( $P < 0.01$ ). In the intestine, where the administration of oil and water sometimes stimulated the lipid phosphorylation, only the difference ( $\bar{C} - \bar{B}$ ) is significant. As for the increases in the average specific activities when oil was given in addition to choline, these increases do not appear to be significant in either the liver or the intestine, except perhaps for the difference ( $\bar{C} - \bar{D}$ ) — ( $\bar{B} - \bar{A}$ ) in the liver which exhibited a low degree of significance ( $0.01 < P < 0.05$ ).

The differences between the average values in Rats A, receiving water, and Rats D, receiving oil and water, are not recorded in Table III. However, for both total radioactivity and specific activity, these differences were found not to be statistically significant.

#### DISCUSSION

The difficulties and complications involved in the calculation of quantitative values for the rate of phospholipid turnover after administration of  $P^{32}$  are well known (1, 9, 14). However, fairly reliable indications concerning *qualitative* changes in this rate can be obtained if the conditions of the experiments are properly chosen. In the present study, the specific activity-time curves of the lipid phosphorus in the liver of Rats A, B, C, and D were determined and, on the basis of the results obtained, a 6 hour interval was adopted for most experiments. This time interval is sufficiently long to minimize the effects of the variations of the specific activity of the inorganic phosphorus in blood (variations which are greatest immediately after the introduction of  $P^{32}$ ), but it still corresponds to the ascending part of the specific activity-time curves, where synthesis of phospholipides in

the liver (or transport to this organ) occurs to a degree greater than their breakdown (or mobilization).

In our experiments, most of the differences between the total radioactivity values were statistically significant, whereas the differences between the specific activities were smaller and usually not significant. This apparent discrepancy probably depends upon the fact that the increases in the total radioactivities were accompanied often by increases in the lipid phosphorus values, indicating that the rate of destruction (or mobilization) of phospholipides was not increased to the same extent as their formation. However, the increases in the lipid phosphorus were proportionately smaller than those in the total radioactivity. This was not unexpected, since the radioactivity values merely express the phospholipides which have been synthesized after the introduction of  $P^{32}$ , whereas the lipid phosphorus figures include both the newly formed phospholipides and those which had been formed earlier. One might thus understand that, while changes in the specific activity have been actually detected in many experiments, these changes were less consistent and less extensive (and, therefore, exhibited a lower degree of statistical significance) than the changes in the total radioactivity. Other points which may also be important in this respect are mentioned in foot-notes 5 and 6.

At any rate, under the conditions of our experiments, an increase in the formation of phospholipides may be reasonably assumed when higher values for total radioactivity are found, and the specific activity values are also higher, or, at least, not lower than in the control animals. Accordingly, our present data confirm the stimulation of lipid phosphorylation by a single dose of choline, which has been previously described in the liver of rats on high fat diets (2, 4). It has now been shown that this stimulation occurs also in animals maintained on a low fat diet, but that it is markedly increased by the simultaneous administration of a large amount of fat.

The latter finding appears to be in line with several previous observations, such as the temporary increase of phospholipides in the liver of dogs during the absorption of a fatty meal (15) and the increased formation of phospholipides in the isolated liver, when lipemic blood was used for the perfusion of the organ (16). An even more direct comparison can be made between our present results and those of the study already mentioned, in which the rates of lipid phosphorylation in the liver of rats on high fat and low fat diets were compared (1). In that study, as pointed out previously, the diets were probably not deficient in choline; moreover, no "depletion" period preceded the experiments. If it is assumed that the supply of choline or choline precursors may act as a limiting factor for the formation of phospholipides, the observations quoted above can be easily reconciled with our present finding that in rats on a choline-deficient diet the adminis-

tration of fat without choline did not increase the formation of phospholipides in the liver.

In recent experiments, Bollman and Flock (17) also failed to find significant differences between the rates of lipid phosphorylation in the livers of rats maintained for 2 or 3 weeks on two low protein diets, one containing 13 per cent, the other 43 per cent of fat. Supplementation of these diets with choline caused an increased formation of phospholipides, but apparently to the same extent in both groups of animals. On this point, we would like to remark that changes in the rate of the synthesis of tissue constituents should be greater and therefore more easily detectable after administration of massive doses and in "acute conditions," as in our experiments. In experiments of longer duration and in which smaller amounts of the active substances are ingested in divided doses, one might expect that the rate differences will be less considerable and possibly within the limits of error in the measurements or within the individual variations of the animals. It is obvious, however, that a relatively slight change in the rate of synthesis of a compound may lead in time to quite marked differences in the tissue composition.

In this respect, it seems of interest to compare our present findings with the results of previous determinations of the amounts of phospholipides in the liver of rats maintained for 19 days on 10 per cent protein diets which contained variable proportions of fats (18). The level of both total and choline-containing phospholipides in the liver was uniformly low. When diets with a low fat content were supplemented with choline, the lecithin level was only slightly increased, whereas when choline was added to diets containing 20 per cent or more of fats, the values for the choline-containing phospholipides were elevated to a marked degree.<sup>6</sup>

The results of our present experiments on the liver of rats on the stock diet are rather inconclusive. It was thought that the drop in the phospholipide content of the liver, which was previously noted when rats on the stock diet were transferred to experimental diets (12), could possibly be

<sup>6</sup> In this investigation the increase in lecithins was accompanied by a decrease in the non-choline phospholipides. Similar findings had been obtained in weanling rats on both low and high fat diets supplemented with choline (19). Moreover, it has been reported recently that in the liver of dogs injected with  $P^{32}$  the administration of choline increases the specific activity in the choline-containing phospholipides, whereas in the non-choline containing fraction the specific activity is definitely lowered (20). The last observation may suggest another explanation for our present finding that after giving choline or choline and fat the increases in the specific activity values are often not marked and exhibit a low degree of statistical significance. Indeed, it seems likely that, also in our experiments, the stimulation of lipid phosphorylation in the liver by choline, or choline and fat, is essentially the expression of an increased formation of choline-containing phospholipides.

due to a decreased rate of phosphorylation in this latter condition. This possibility cannot be excluded (since the decrease may be small enough not to be demonstrable; see above), but it is certainly not favored by the comparison between the data obtained in Rats A on the stock diet and in Rats A on the choline-deficient diet, respectively. Indeed, lower values for the specific activity were mostly found in the former animals.

Our stock diet contains approximately the same proportion of fat (5 per cent) as Diet 26, but is richer in choline and in proteins containing choline precursors. One may understand that in the rats maintained on the stock diet the administration of additional amounts of choline was only slightly, or not at all, effective. However, one would have expected that, after giving choline and fat, or even fat only, an increase in the rate of lipide phosphorylation would be detected in the liver (as it has been found in the previous and present determinations on the intestine of rats on the stock diet). Actually, only the results of one experiment correspond to this expectation. While we have no simple explanation to offer for this discrepancy, it should be pointed out that, besides the content of the diet in fat and choline (or choline precursors in the form of proteins), a number of factors, intrinsic and extrinsic, are possibly involved in the formation of liver phospholipides. Thus, in previous investigations on the phospholipide composition of the liver of rats on experimental diets, it was noted that the effects of choline administration were modified, to a more or less marked degree, by the age of the animal (19), by a previous period of "depletion" on the deficient diet (8), and by the nature of the carbohydrate component of the diet (21). Other known and unknown constituents of mixed natural diets could also be important. Their presence in the stock diet may well be responsible for the discrepancy between the results obtained in animals on this diet and those which we would have expected on the basis of experiments in which diets with a simpler and better defined composition were employed.

Aside from the findings on the animals on the stock diet, in the rats on the low fat, low choline diet the effects of the administration of choline and fat on the formation of phospholipides seem to be substantially identical in the liver and in the intestine. The results of the additional determinations which have been made on the lipides of this organ are in general agreement with those reported previously (6), except that in the intestine of rats on the deficient diet the administration of oil, even without choline, sometimes did cause a definite increase in both total activity and specific activity. Several explanations might be postulated for these occasional findings, such as the existence of differences in the degree of choline depletion, or in the extent of the synthesis of choline by the tissues, possibly also by the intestinal flora (22).

## SUMMARY

1. Rats maintained for 7 days on a low protein, low fat diet, were given by stomach tube water, or choline, or oil and choline, or oil and water. The animals were then injected with a solution of inorganic phosphate containing radioactive phosphorus. After certain time intervals, the total radioactivity and the phosphorus content were determined in the lipides of the liver.

2. The administration of a single dose of choline stimulated the lipide phosphorylation in the liver. This effect was definitely enhanced by the simultaneous ingestion of a large amount of fat. The administration of oil only was ineffective.

3. These findings closely resemble those previously obtained in similar experiments on the small intestine. Additional evidence for the action of choline and fat on the formation of phospholipides in this tissue is also presented.

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## STUDIES ON BIOLOGICAL OXIDATIONS

### XXI. THE METABOLISM OF LUNG AS DETERMINED BY A STUDY OF SLICES AND GROUND TISSUE

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The literature on the metabolism of the lung is very scanty. There are scattered observations on the metabolism of a particular substance by either the sliced or ground tissue, and on the presence of some enzyme in lung, but there is no systematic study of its biochemical activities. Furthermore, some of these observations were made on ground lung tissue (1) without comparison of the results thus obtained with observations on tissue slices. On rupturing cell membranes and on destroying the cellular architecture there occurs not only destruction of some biochemical activities but also appearance of others normally inhibited in the intact cell. Studies on the metabolism of the lung, as performed in slices and in ground tissue, are presented in this paper.

#### EXPERIMENTAL

The lung slices were made free-hand with thin razor blades and were received in ice-cold Ringer-phosphate buffer. The ground tissue was prepared by being ground first in a mortar cooled at 0°, and then homogenized in the Potter and Elvehjem homogenizer (2). O<sub>2</sub> uptake and CO<sub>2</sub> formation were measured with the usual manometric technique of Warburg. Respiratory quotients were determined according to the method of Warburg and Yabusoe (3). Lactic acid was determined colorimetrically according to the method of Miller and Muntz (4). NH<sub>3</sub> was determined by a modification of the distillation technique of Conway (5). Pyruvic and  $\alpha$ -ketoglutaric acids were determined by the method of Friedemann and Haugen (6). Citric acid was determined colorimetrically by the method of Pucher *et al.* (7). Diphosphopyridine nucleotide (DPN) was determined by a modification of the method of Jandorf, Klemperer, and Hastings (8). In this method, based on the enzymatic conversion of hexose diphosphate into phosphoglyceric and glycerophosphoric acids by an extract of muscle acetone powder and DPN, it is necessary to destroy the DPN remaining in the muscle powder. Jandorf *et al.* used for this purpose charcoal adsorption. We found this procedure unreliable and we have replaced it by the enzymatic destruction of DPN with ground lung tissue which is extremely



rich in nucleosidase. The ground, homogenized muscle is mixed with ground lung tissue and incubated for 30 minutes at 38°, after which it is centrifuged. The supernatant fluid is then treated according to Jandorf *et al.* Succinic acid was determined manometrically with pigeon breast succinoxidase.

*Respiration of Lung Slices and Ground Lung*—The respiration of lung slices from adult rats gave values as reproducible as those obtained with other tissues of the body. The  $QO_2$  (c.mm. of  $O_2$  uptake per mg. of dry

TABLE I  
*Respiration of Rat Lung Slices*

Buffer, Ringer-phosphate; pH, 7.4;  $O_2$  as gas phase; temperature 38°; no substrate.  $QO_2$  = c.mm. of  $O_2$  uptake per mg. of dry tissue per hour. Each experiment is the average of three determinations.

Animal No.	Slice, $QO_2$	Ground tissue, $QO_2$
1	6.78	0.68
2	8.23	0.77
3	6.53	0.81
4	7.68	0.71
5	7.67	0.80
6	8.58	0.77
7	8.11	0.73
8	7.17	0.75
9	8.97	0.65
10	8.58	0.85
11	6.18	
12	7.11	
13	8.44	
14	7.88	
15	7.66	
16	7.70	

weight per hour) was  $7.70 \pm 0.60$  (Table I). This value is in agreement with that obtained by Laser, 7.8 (9). The  $O_2$  uptake of the ground lung, measured immediately after grinding and soon after temperature equilibration of the manometers (12 minutes), was  $0.75 \pm 0.05$ . This 10-fold drop in respiration of the ground tissue is remarkable and has no counterpart in any other tissue.

The rate of  $O_2$  uptake of the slices was kept as constant as that of any other tissue slice, as can be seen in the experiment reported in Fig. 1. In contrast with this constant rate obtained in slices, the respiration of ground tissue fell abruptly from the 1st hour, ceasing completely at the end of the

3rd hour. It is well known that the  $O_2$  uptake of ground tissue falls rapidly, although in some instances, as in muscle, a steady state could be maintained on addition of small amounts of fumarate (10). In ground lung, fumarate addition had no effect at all.

The respiratory quotient of rat lung slices was 0.85 (Table II).

*Species Difference*—In Table III are given data for the  $O_2$  uptake of lung slices of different animal species. In these experiments the  $O_2$  uptake was

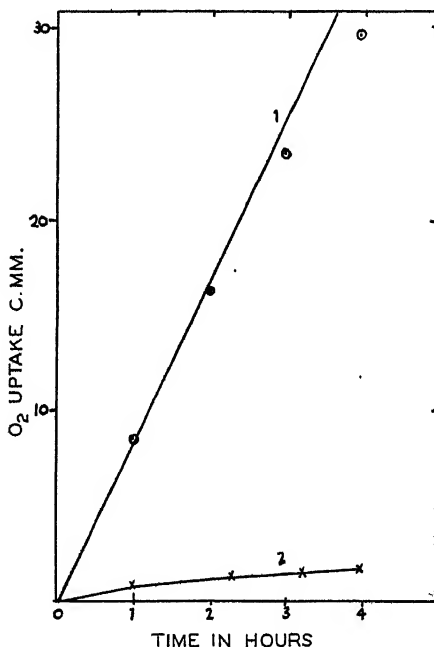


FIG. 1. Respiration of lung slice and ground lung.  $O_2$  uptake per mg. of dry weight. Curve 1, lung slice; Curve 2, ground lung.

measured with and without succinate. The  $QO_2$  values varied as follows: rat > rabbit > guinea pig > cat > pigeon. The addition of succinate increased the  $O_2$  uptake by 30 per cent in the rat, 26 per cent in the cat and pigeon, and 17 per cent in the rabbit lung.

*Utilization of Oxidizable Substrates*—There are animal tissues, such as the kidney and the heart, whose basal  $O_2$  uptake is greatly increased on addition of oxidizable substrates. Others, like the liver, are not very much affected. The  $O_2$  uptake of lung slices was not altered by the addition of glucose, hexose-6-monophosphate, hexose diphosphate, citrate, fumarate, malate, pyruvate,  $\alpha$ -ketoglutarate, and histidine. Alanine and acetate produced a small increase. The only substrates producing a definite increase on the

O<sub>2</sub> uptake were lactate, succinate, aspartate, histamine, and  $\beta$ -hydroxybutyrate (Table IV). This failure of a large number of oxidizable substrates to increase the O<sub>2</sub> uptake of lung slices was not always due to lack of utilization, for glucose,  $\alpha$ -ketoglutarate, pyruvate, citrate, alanine, glutamate, and aspartate were utilized, as was demonstrated by chemical analysis (Table V). Although utilization of these substrates was rather low when compared to utilization by the kidney or the liver, it compares well with

TABLE II  
*Respiratory Quotient of Lung (Rat Slices)*

Ringer-phosphate, pH 7.4; O<sub>2</sub> as gas phase; substrate, glucose, 0.01 M; temperature 38°.

Rat No.	QO <sub>2</sub>	QCO <sub>2</sub>	R.Q.
1	7.05	6.34	0.90
2	7.80	6.71	0.86
3	7.72	6.42	0.83
4	7.86	6.61	0.84
5	7.70	6.32	0.82
Average .....	7.62	6.48	0.85

TABLE III  
*Respiration of Lung (Slices); Species Difference*

Buffer, Ringer-phosphate, pH 7.4; succinate, 0.01 M; Q succinate, c.mm. of succinate utilized per mg. of dry weight per hour.

Species	QO <sub>2</sub>	QO <sub>2</sub> succinate	Q succinate
Rat.....	7.70	10.0	
Rabbit.....	6.68	7.86	2.02
Guinea pig.....	6.09		
Cat.....	3.86	4.89	2.11
Pigeon.....	3.61	4.54	3.38

that observed in the prostate (11). Pyruvate utilization was greater in the presence of O<sub>2</sub> than in N<sub>2</sub>-CO<sub>2</sub>, giving an oxidismutation coefficient (pyruvate utilized in O<sub>2</sub>-CO<sub>2</sub>)/(pyruvate utilized in N<sub>2</sub>-CO<sub>2</sub>) of 1.3. Utilization of histamine was high when compared to that of amino acids or carboxylic acids. There was no NH<sub>3</sub> formation from cysteine or cystine.

*Glycolysis*—Laser (9) reported for the anaerobic glycolysis of lung slices a Q<sub>CO<sub>2</sub></sub><sup>N<sub>2</sub></sup> value of 1 (c.mm. of CO<sub>2</sub> produced per mg. of dry weight per hour). Simon *et al.* (12) gave values between 1.5 and 2.25. The value found in this laboratory was higher, namely 4.54. The aerobic glycolysis,

as measured by lactic acid determination, was 1.94 (Table VI). Whether this value for the aerobic glycolysis was partly due to the glycolysis of the blood contained in the tissue was not determined. The ground tissue lost the ability to ferment glucose, as is shown by the lack of lactic acid formation whether aerobically or anaerobically.

*Oxidations Produced by Ground Lung Tissue*—The  $O_2$  uptake of ground lung tissue was increased on addition of succinate, histamine, choline, and tyramine (Fig. 2). Substances which were oxidized by lung slices, such as

TABLE IV  
*Effect of Oxidizable Substrates on Oxygen Uptake of Lung (Rat Slices)*  
Buffer, Ringer-phosphate, pH 7.4; substrate concentration, 0.01 M.

Substrate	QO <sub>2</sub> values		Increase  per cent
	No substrate	With substrate	
Glucose.....	7.68	7.58	None
Hexose-6-monophosphate.....	7.68	7.49	"
Hexose diphosphate.....	7.68	7.55	"
Lactate.....	7.67	9.52	24
Malate.....	7.67	8.00	None
Citrate.....	7.70	7.80	"
Fumarate.....	8.52	8.49	"
Succinate.....	7.67	10.02	30.5
Pyruvate.....	8.23	8.06	None
$\alpha$ -Ketoglutarate.....	8.23	8.36	"
DL-Alanine.....	6.78	7.48	10.3
Aspartate.....	6.78	7.88	16
Glutamate.....	6.78	8.07	19
Histidine.....	7.17	7.50	None
Histamine.....	7.17	8.44	18
$\beta$ -Hydroxybutyrate.....	8.11	9.29	14.5
Acetate.....	7.88	8.62	8.5

pyruvate, lactate, aspartate, were untouched by the ground tissue. One of the factors for the loss of oxidative power seems to be destruction of DPN. This was shown in experiments with  $\beta$ -hydroxybutyric acid: the  $O_2$  uptake remained unaffected on its addition; there was increase of  $O_2$  uptake and oxidation of  $\beta$ -hydroxybutyrate on addition of  $1 \times 10^{-4}$  M DPN.

*Adenosine Deaminase in Lung Tissue*—Conway and Cook (13) found that tissue extracts deaminated adenylic acid without previous dephosphorylation. According to these investigators, the appendix, jejunum, and spleen had the highest adenosine deaminase activity, the kidney, brain, and lung had 20 per cent of the activity of spleen, the liver 10 per cent,

while the skin had no adenosine deaminase. Lung tissue has been found as active as spleen. In fact, from all the tissues studied in the rat, lung,

TABLE V

*Utilization of Some Oxidizable Substrates by Rat Lung (Slices)*

Buffer, Ringer-phosphate, pH 7.4; gas phase,  $O_2$ ; substrate concentration, 0.01 M. The figures represent c.mm. of substrate utilized per mg. of dry weight per hour.

Substrate	Utilization
	<i>c.mm.</i>
Citrate.....	1.60
$\alpha$ -Ketoglutarate.....	0.90
Pyruvate (in $O_2$ - $CO_2$ ).....	4.29
" (" $N_2$ - $CO_2$ ).....	3.29
<b><math>NH_3</math> formation</b>	
No substrate.....	0.97
DL-Alanine.....	1.65
Aspartate.....	2.05
Glutamate.....	1.33
Histidine.....	0.99
Histamine.....	2.22

TABLE VI

*Glycolysis of Rat Lung (Slices)*

The figures represent c.mm. of  $CO_2$  or lactic acid produced by 1 mg. of dry tissue per hour in the presence of Ringer-bicarbonate with  $N_2$ - $CO_2$  (anaerobic glycolysis) or  $O_2$ - $CO_2$  (aerobic glycolysis) as the gas phase.

Rat No.	Glycolysis	
	Anaerobic	Aerobic
1	3.25	1.80
2	3.90	1.47
3	4.07	2.51
4	5.04	2.50
5	4.89	1.65
6	4.63	1.21
7	4.63	1.70
8	4.99	2.86
9	5.48	1.75
Average.....	4.54	1.94

spleen, kidney, testis, brain, and liver, the lung showed the highest activity (Table VII). In these experiments the tissue was homogenized in the presence of ice-cold water, and deamination was measured in the presence

of 0.01 M adenosine and phosphate buffer (0.05 M), pH 7.4. The incubation time was 10 minutes at 38°.

*Inactivation of Pyridine Nucleotides*—It has been known for some time that pyridine nucleotides are inactivated by tissue suspension. Some of

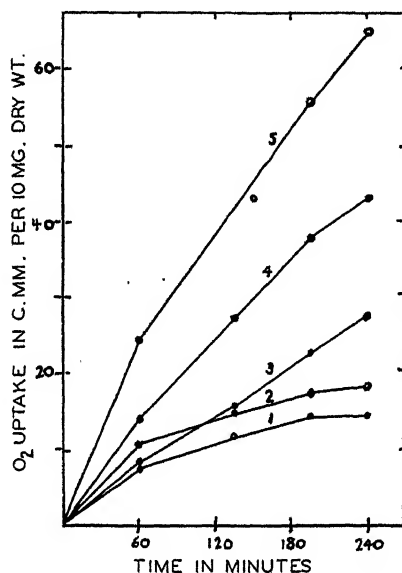


FIG. 2. Oxidations produced by ground lung tissue. Buffer, 0.05 M phosphate, pH 7.4; temperature 38°; substrates, 0.01 M. Curve 1, no substrate; Curve 2, choline; Curve 3, histamine; Curve 4, tyramine; Curve 5, succinate.

TABLE VII

*Deamination of Adenosine by Ground Lung Tissue (Rat)*

Buffer, phosphate, 0.05 M, pH 7.4; adenosine, 0.01 M. The figures represent c.mm. of  $\text{NH}_3$  formation per mg. of dry weight (blank subtracted).

Tissue	$\text{NH}_3$ formation c.mm.	Tissue	$\text{NH}_3$ formation c.mm.
Lung	16.9	Testis	6.2
Spleen	16.9	Brain	4.1
Kidney	11.2	Liver	2.0

the properties of the enzyme which split pyridine nucleotides have been described by Handler and Klein (14), according to whom they are split with liberation of nicotinamide. In order to ascertain that the rapid fall in respiration of ground lung tissue was due to its high content of nucleosidase, the enzymatic inactivation of DPN was studied as follows: Tissues

were homogenized at 0°. Various dilutions of the tissue suspension were then incubated with  $2 \times 10^{-4}$  M DPN for 15 minutes at 38°. At the end of the incubation period, the tubes were rapidly boiled, and DPN was determined by the method of Jandorf, Klemperer, and Hastings (8). Numerous determinations were made in order to obtain the amount of tissue required to destroy half of the DPN added. In Table VIII are given the number of mg. of tissue required to produce this destruction, and the relative activity of the enzyme in the different tissues examined. According to these determinations, lung tissue has the highest concentration of the enzyme per unit weight. This high nucleosidase activity of ground lung tissue has been utilized to destroy the DPN contained in the muscle acetone powder used

TABLE VIII

*Nucleotidase Activity of Ground Tissues (Rat Ground Tissue)*

Measured by the inactivation of diphosphopyridine nucleotide ( $2 \times 10^{-4}$  M) added to the tissue suspensions. The figures represent mg. of tissue required to destroy  $1 \times 10^{-4}$  M pyridine nucleotide.

Tissue	Required for half destruction of DPN	Relative nucleotidase activity
	mg.	
Lung.....	1.2	100
Intestine.....	1.9	63
Brain.....	3.6	33
Liver.....	4.3	28
Heart.....	5.5	22
Kidney.....	8.1	15
Skin.....	100 +	1
Testis.....	No activity	0
Blood.....	" "	0

by Jandorf *et al.* in the determination of DPN. It may be concluded from these experiments that the tremendous fall in respiration due to grinding is due to the release from the cell of the large amounts of nucleosidase present in lung. This is a factor which must be taken into consideration whenever the lung tissue is damaged.

*Carbonic Anhydrase*—It has been reported by van Gor (15) that lung tissue contains no carbonic anhydrase. It is difficult to determine the activity of carbonic anhydrase in lung because of the large amount of red cells and because of the loss of the enzyme on washing the tissue free of blood. Carbonic anhydrase estimations were made by grinding the lung tissue (rat) in water (1 part in 10 of water). The enzyme activity was determined by the "boat" technique of Meldrum and Roughton (16). One side of the boat contained 1.25 cc. of phosphate buffer, 0.2 M, pH 6.3, 0.1 cc.

of the lung suspension, and 0.15 cc. of  $\text{H}_2\text{O}$ . The other side contained 1.5 cc. of the  $\text{NaHCO}_3$ - $\text{NaOH}$  mixture (0.2 M  $\text{NaHCO}_3$  in 0.04 M  $\text{NaOH}$ ). The vessels were kept at  $1.8^\circ$ . After temperature equilibrium the contents of both sides of the vessels were mixed (they were shaken at a rate of 88 excursions per minute), and readings in the manometer were made to 1 and 2 cm. excursions. In this manner, the  $\text{CO}_2$  formation of the blank, of the tissue, and of a hemoglobin solution from rat blood (equal to the hemoglobin content of lung) was measured. Carbonic anhydrase activity was measured by applying Meldrum and Roughton's formula  $(R - R_0)/R_0$ , where  $R_0$  is the reciprocal of the time (seconds) taken for the second cm. of the

TABLE IX  
*Acetylcholine and Tributyrin Esterase of Tissues*

Tissues, ground in a mortar (1 mg., dry weight). Buffer, Ringer-bicarbonate, pH 7.4; substrate, 0.03 mm; time, 30 minutes; temperature  $38^\circ$ .

Tissue	$\text{CO}_2$ output		Ratio of acetylcholine to tributyrin
	Tributyrin	Acetylcholine	
	<i>c.mm.</i>	<i>c.mm.</i>	
Brain.....	28.5	54	1.9
Heart.....	34.2	19.5	0.57
Serum.....	50	15	0.3
Spleen.....	21	9.8	0.46
Kidney.....	260	40	0.153
Lung.....	56	7.5	0.13
Liver.....	530	3	0.005
Testis.....	67	0	0

manometer excursion (due to  $\text{CO}_2$  evolution) in the absence of enzyme, and  $R$ , the same in the presence of enzyme. In lung tissue these values were 8.7 (per mg. of tissue), while the blood contained in the tissue gave a value of 1.7.

*Esterase Activity*—It is difficult to distinguish in ground tissue non-specific esterase from cholinesterase. The experiments reported in Table IX, in which the hydrolysis of tributyrin (non-specific) and acetylcholine was determined simultaneously, show that, while in brain tissue the ratio of tributyrin hydrolyzed to acetylcholine hydrolyzed was 10:19, in the lung the ratio was 10:1.3. It seems that lung tissue contains no cholinesterase.

#### SUMMARY

The oxygen consumption of lung slices was remarkably constant in adult rats, the  $\text{QO}_2$  being  $7.70 \pm 0.60$ . An almost steady rate was maintained, and at the end of the 4th hour it had dropped only by 25 per cent.



The  $QO_2$  values of ground tissue, on the other hand, were only one-tenth that of slice and at the end of the 3rd hour the  $O_2$  uptake stopped. One of the most important factors for this fall in respiration seems to be the release of nucleosidase; in fact, lung tissue was found to be the richest in this enzyme of all tissues examined. In the intact cell this enzyme is kept out of contact with pyridine nucleotides, as is shown by the fact that in tissue slices are found biochemical reactions requiring pyridine nucleotides, whereas in the ground tissue these reactions (glycolysis, utilization of lactate, glutamate,  $\beta$ -hydroxybutyrate) did not occur. This sudden release of nucleosidase on damage of the lung might play a considerable rôle in the mechanism of action of some lung irritants.

The  $O_2$  uptake of lung slices was increased above 10 per cent only by succinate, histamine,  $\beta$ -hydroxybutyrate, glutamate, aspartate, lactate, and alanine. The  $O_2$  uptake of ground lung tissue was increased only by succinate, hexose monophosphate, histamine, tyramine, and choline.

The anaerobic glycolysis of lung slices was  $Q_{CO_2}^{N_2}$  4.54; the aerobic glycolysis, 1.94. Ground tissue showed no glycolysis.

The  $O_2$  uptake of lung tissues in different animal species had values decreasing as follows: rat > rabbit > guinea pig > cat > pigeon. Adenosine deaminase was found abundant in lung tissues. Carbonic anhydrase was also present.

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## STUDIES ON BIOLOGICAL OXIDATIONS

### XXII. THE METABOLISM OF THE BONE MARROW\*

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A relatively small amount of work has been done on the metabolism of the bone marrow. Van Breza (1) in 1926 first measured the  $O_2$  uptake of teased preparations of rabbit bone marrow and reported  $QO_2$  values of 3.5 to 5.0, calculated on a fat-free, dry weight basis. Fujita (2) found an initial  $QO_2$  value of 12.9, falling to 0 in 3 hours in suspensions of cells separated from the marrow by shaking it in Ringer-phosphate buffer. Orr and Stickland (3) reported an average  $QO_2$  of 3.9. Warren (4) reported a  $QO_2$  value of 6.1 in serum and 3.7 in Ringer-phosphate solution. In a series of papers since 1940, Warren studied the effects of respiration and glycolysis of variations in cellular components (5), of lowered oxygen tension (6), of potassium arsenite (7), and of thiouracil (8).

The bone marrow is the site of great cellular activity because cells are continuously being formed in this tissue. We have chosen it for this reason for the study of condensation reactions leading to synthesis. It was necessary, however, to determine the rate and extent of enzymatic reactions which presumably are used to provide energy for these synthetic reactions. We present in this paper data on the utilization of oxidizable substrates and on the normal content of some coenzymes.

#### EXPERIMENTAL

The bone marrow was obtained from normal, healthy adult rabbits weighing about 2 kilos. The animals were killed by decapitation and the marrow was removed as a sausage from the femora, humeri, and tibiae. The sausages were sliced free-hand, minced with a razor, ground in a loose glass homogenizer, and shaken to disintegration or teased in Ringer-phosphate solution.

Because of the great variation in fat content of bone marrow it is impossible to use wet weight or dry weight as a basis for calculation of metabolic activity. Orr and Stickland (3) determined the ratio of nitrogen to fat-free dry weight of normal marrow and reported a value of 14.6 per

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cent. In the experiments reported here, wet weights were measured either directly on a watch-glass or by adding the tissue to a tared tube containing a weighed amount of water. The tissue was then thoroughly ground in a glass homogenizer and aliquots taken for fat-free dry weight and nitrogen analysis. For the former determination, a 1 cc. aliquot was transferred to a tared centrifuge tube and dried in the oven overnight at 105°. The residue was extracted once with 10 cc. and twice with 5 cc. of petroleum ether. Care was taken to pulverize thoroughly the tissue with a glass pestle during the extractions. The petroleum ether was removed each time by centrifugation at 3° and the residue was weighed after drying to constant weight. Nitrogen determinations were made upon a similar

TABLE I

*Relation between Wet Weight, Fat-Free Dry Weight, and Total Nitrogen in Normal Rabbit Bone Marrow*

All samples started with a wet weight of 1000 mg.

Experiment No.	Fat-free dry weight	Total nitrogen	Nitrogen
			Fat-free dry weight $\times 100$
	mg.	mg.	
1	126.5	14.4	11.42
2	102.0	12.1	11.86
3	103.2	12.5	12.10
4	125.8	14.6	11.10
5	144.3	14.8	10.35
6	142.5	15.8	11.12
7	101.0	12.1	11.97
Average.....	121 $\pm$ 16	13.8 $\pm$ 1.3	11.42 $\pm$ 0.49

aliquot. The results of one such series of experiments are shown in Table I. The figure for nitrogen to fat-free dry weight  $\times 100$  of 11.42 is lower than that obtained by Orr and Stickland. It was decided to express the results first in relation to total nitrogen and convert the result afterwards to fat-free dry weight to obtain the customary  $QO_2$  values. Diphosphopyridine nucleotide was determined by the method of Jandorf *et al.* (9) as modified by Barron *et al.* (10). Diphosphothiamine was determined by the method of Lohmann and Schuster (11) as modified by Ochoa and Peters (12).

*Bone Marrow Respiration*—The respiration of the bone marrow, like that of other tissues, is decreased on grinding the tissue. In the presence of Ringer-phosphate buffer the  $QO_2$  value of slices was 3.29, while that of minced tissue (bone marrow teased with forceps and dispersed by shaking in Ringer's solution) was 2.10 (Table II). Furthermore, the respiration

of slices remained constant for at least 2 hours, while that of minced tissue was constant for only half an hour. In Table III there are given data on the respiration of bone marrow slices suspended in Ringer-phosphate solution and in the phosphate buffer prepared according to Orr and Stickland. The  $QO_2$  values in the latter buffer were slightly higher than those in Ringer-

TABLE II  
*O<sub>2</sub> Uptake of Bone Marrow; Sliced and Minced Tissue*

$QO_2$ values	
Slice	Minced tissue
2.75	2.01
3.96	2.43
4.02	2.76
2.53	1.62
3.19	1.67
Average..... 3.29	2.10

TABLE III  
*O<sub>2</sub> Uptake of Bone Marrow Suspended in Ringer-Phosphate Solution and in Orr's Phosphate Buffer, Both Containing 0.01 M Glucose*

$QO_2$ values	
Ringer-phosphate	Orr's buffer
2.20	2.67
2.74	3.14
2.67	3.74
4.25	4.01
3.90	3.47
3.87	4.08
2.78	3.79
2.72	2.97
	3.79
	3.47
	3.44
Average..... $3.14 \pm 0.65$	$3.51 \pm 0.34$

phosphate solution. All succeeding respiration experiments were performed with bone marrow slices in the Orr and Stickland buffer.

*Utilization of Substrates by Bone Marrow*—The  $O_2$  uptake of bone marrow slices did not increase on addition of glucose (0.01 M). The anaerobic glycolysis was inhibited by glyceraldehyde (0.005 M) by 50 per cent during the 1st hour and by 70 per cent in the 2nd hour (Fig. 1), an indication that

glycolysis in bone marrow is glucose glycolysis instead of glycogen glycolysis.

Addition of pyruvate produced no effect on the  $O_2$  uptake, although chemical analysis showed utilization. In the presence of  $O_2$  the  $Q$  pyruvate value was 1.61 with a lactate formation of 0.16. The utilization of pyruvate in the absence of  $O_2$  ( $N_2$  as gas phase), which may be considered as a dismutation process, was 0.75, with a lactate formation of 0.28. These values would give an oxidismutation coefficient for pyruvate utilized in  $O_2$  to pyruvate utilized in  $N_2$  of 2.12 (Table IV), which is higher than the values for other tissues reported by Barron (13). These experiments are

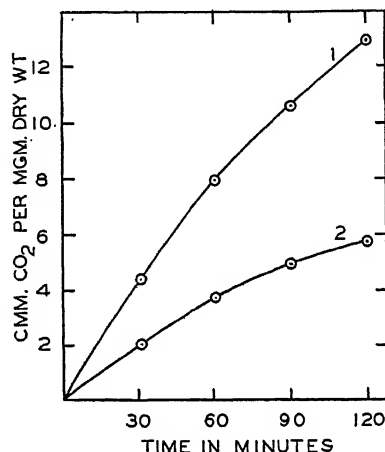


FIG. 1. Effect of glyceraldehyde on anaerobic glycolysis of rabbit bone marrow slices. Curve 1, control; Curve 2, control + 0.005 M glyceraldehyde.

an indication that the main pathway of pyruvate metabolism is the oxidative pathway.

Lactate (0.01 M) had no effect on the  $O_2$  uptake.

The respiration of bone marrow slices was increased by about 21 per cent on addition of acetate (0.01 M). This increase in the  $O_2$  uptake was accompanied by utilization of acetate, the average  $Q$  acetate being 1.89 which is remarkably high when compared with the total  $O_2$  uptake, 4.56 (Table V). Further proof of acetate metabolism by the bone marrow was obtained by the inhibition of its respiration with fluoroacetate, a halogen acid which was found by Bartlett and Barron (14) to be a specific inhibitor of acetate oxidation. The  $O_2$  uptake of bone marrow in Ringer-phosphate solution was inhibited by fluoroacetate 48 per cent in the 1st hour and up to 65 per cent in the 3rd hour. This inhibition increased in the presence of acetate (Fig. 2). Utilization of acetate was completely inhibited by fluoroacetate. Moreover, there was an accumulation of acetate of 0.41 cmm. per mg. of

dry weight. Fluoroacetate (0.01 M) inhibited 45 per cent the utilization of pyruvate by the bone marrow, in agreement with similar findings of

TABLE IV

*Pyruvate Metabolism by Bone Marrow*

Figures in c.mm. per mg. of fat-free dry weight per hour.

QO <sub>2</sub>	Q pyruvate in O <sub>2</sub>	Q pyruvate in N <sub>2</sub>	Lactate formation	
			In O <sub>2</sub>	In N <sub>2</sub>
	0.96	0.590	0.127	0.259
	1.15	0.775	0.148	0.263
	1.48	1.13		
	1.47	0.954		
3.99	2.00	0.605		
3.67	1.21	0.666		
3.28	1.52			
3.15	1.59	0.540		
	1.61			
	1.75	0.915		
3.50	1.04			
3.42	1.08			
3.40	2.54	1.17	0.260	0.310
3.50	2.28	0.768	0.226	0.361
3.42	1.87	0.351	0.121	0.280
3.82	2.18	0.545	0.074	0.224
Average. 3.51	1.61	0.75	0.16	0.28

TABLE V

*Acetate Metabolism by Bone Marrow*

Acetate concentration, 0.01 M. Figures in c.mm per mg. of fat-free dry weight per hour.

QO <sub>2</sub>	QO <sub>2</sub> acetate	Q* acetate
3.72	4.10	-2.18
3.70	4.32	-1.77
4.50	4.71	-2.23
4.00	5.22	-1.38
3.40	4.75	
3.30	4.25	
Average. 3.77	4.56	-1.89

\* Q acetate values were obtained in independent experiments.

Bartlett and Barron (14) in the kidney and liver. Other fatty acids increased the respiration of bone marrow, an indication of the important rôle of fatty acid metabolism in this tissue.

Bartlett and Barron (14) have given evidence in favor of the view that pyruvate, and hence carbohydrate, in the presence of oxygen, is utilized mainly via pyruvate oxidation to acetate. Further oxidation would then proceed through Krebs' tricarboxylic acid cycle, starting with the condensation of acetate and oxalacetate to give citrate or isocitrate. In Table VI data are given on the effect of the different substances formed in this cycle on the  $O_2$  uptake of bone marrow. Citrate and  $\alpha$ -ketoglutarate had no effect, although there was utilization of these substrates: 0.3 c.mm. of citrate per mg. per hour and 0.13 c.mm. of  $\alpha$ -ketoglutarate.

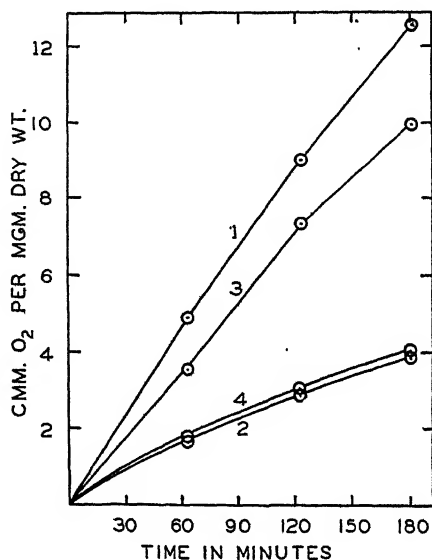


FIG. 2. Effect of fluoroacetate on the oxygen uptake of rabbit bone marrow with and without acetate. Curve 1, acetate (0.01 M); Curve 2, acetate + fluoroacetate (0.01 M); Curve 3, control; Curve 4, fluoroacetate.

Fumarate and malate had small effect. The maximum increase was found on addition of succinate and of oxalacetate + acetate.

Bone marrow contained very little citric acid (8  $\gamma$  per mg. of N, *i.e.* 1  $\gamma$  per mg. of dry weight). On incubation in the presence of oxalacetate (0.02 M) and  $O_2$  as gas phase there was citric acid synthesis (0.6 c.mm. per mg. of fat-free dry weight per hour). In the presence of oxalacetate and pyruvate (0.01 M) there was some increase in citric acid formation (0.8 c.mm. per mg. per hour). Such an increase was not observed when magnesium acetate (0.01 M) replaced pyruvate (0.7 c.mm.). Bone marrow contained aconitase, as can be seen in the experiment plotted in Fig. 3 where the

formation of citric acid from *cis*-aconitic acid was measured. The *Q* aconitase value, 6.9 (c.mm. of citric acid formed per hour per mg. of dry weight)

TABLE VI  
*Effect of Substances Belonging to Krebs' Cycle on O<sub>2</sub> Uptake of Bone Marrow (Slices)*

All the substrates were added to give 0.01 M. Buffer, Orr and Stickland Ringer-phosphate solution.

Substance	QO <sub>2</sub>	Increase	Q substrate
	c.mm.	per cent	c.mm.
None .....	3.8		
Citrate .....	3.9	None	0.3
$\alpha$ -Ketoglutarate .....	3.9	"	0.13
Succinate .....	5.35	41	
Succinic semialdehyde .....	4.35	14	
Fumarate .....	4.05	6	
Malate .....	4.05	6	
Oxalacetate .....	5.1	34	
" + pyruvate .....	4.85	28	
" + acetate .....	5.4	42	
Acetate .....	4.6	21	1.8

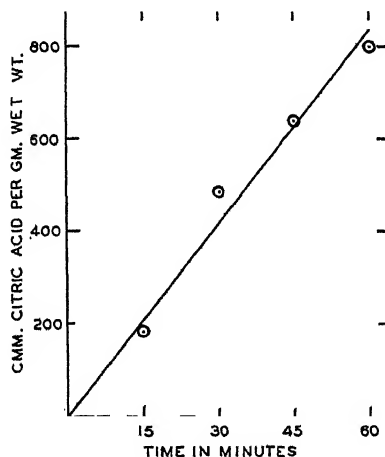


FIG. 3. Aconitase in rabbit bone marrow. 1 gm. of marrow ground with 5 volumes of phosphate buffer (0.1 M), diluted to 40 cc., and centrifuged. 4 cc. of supernatant + 4 cc. of Ringer's solution + 4 cc. of 0.1 M phosphate buffer + 4 cc. of 0.1 M sodium *cis*-aconitate incubated at 38° for 1 hour. Aliquots withdrawn at 15 minute intervals for citrate analysis.

is about that of testis, reported by Johnson (15). The cytochrome oxidase content of bone marrow found by Schultze (16) has been confirmed in this laboratory.



That the metabolism of bone marrow goes in part through the Krebs' tricarboxylic acid cycle is indicated by the inhibition produced by malonate. Penetration of malonate through cellular membranes was demonstrated by complete inhibition of the extra  $O_2$  uptake due to succinate oxidation. We have no explanation for the small inhibition produced with oxalacetate, an inhibition which was less than that produced on the  $O_2$  uptake of tissue with no substrate (Table VII).

Amino acid metabolism seemed to be low in the bone marrow. Glutamate and aspartate had the same lack of effect on the  $O_2$  uptake, and a very small amount of  $NH_3$  was formed in the experiments with glutamate (0.02 c.mm. per mg. of fat-free dry weight per hour). There was no  $NH_3$  formation in the absence of glutamate.

No choline oxidase was found in ground, washed bone marrow suspensions, a finding which is surprising because of the important rôle of choline in some of the functions of bone marrow.

TABLE VII  
*Malonate Inhibition of Bone Marrow Metabolism*

Malonate concentration, 0.03 M; substrate, 0.01 M.

Substrate	QO <sub>2</sub> malonate	QO <sub>2</sub> control	Inhibition
	c.mm.	c.mm.	per cent
None .....	2.94	4.25	31
Acetate .....	3.06	4.32	30
Oxalacetate .....	3.72	4.46	16
Succinate .....	3.14	5.53	43

*Diphosphopyridine Nucleotide (DPN) and Diphosphothiamine*—For the estimation of DPN, the marrow was placed immediately in water heated to 90°. After heating for 5 minutes the tissue was homogenized, centrifuged, and the supernatant fluid was used for analysis of DPN. The low values obtained suggested the presence in the tissue of a nucleotidase, which was confirmed by experiments in which DPN destruction by ground bone marrow was observed. When the marrow was placed in 1 per cent nicotinamide immediately after removal from the bone, as recommended by Mann and Quastel (17), values 3 to 4 times as high were obtained (Table VIII). The average value, 121  $\gamma$  per gm. of fresh tissue, is higher than that of erythrocytes (100  $\gamma$ ) and approaches that of kidney (160  $\gamma$ ).

The results of a series of determinations of diphosphothiamine are shown in Table IX. The average value of 2.26  $\gamma$  per gm. of fresh tissue, the lowest figure of all tissues, could not be increased by modifying the techniques of extraction.

A few experiments were performed with erythroid marrows (phenylhydrazine poisoning) and granulocytopenic marrows (produced with nitrogen mustards). Both seemed to have lower values of DPN.

*Effect of Serum and Boiled Yeast Juice on Bone Marrow Respiration*—Warren (4) showed that the respiration of bone marrow was increased when suspended in serum. The supernatant fluid obtained from serum heated

TABLE VIII  
*Diphosphopyridine Nucleotide Content of Bone Marrow in Presence and in Absence of Nicotinamide*

With nicotinamide		No nicotinamide	
Per gm. fresh tissue	Per mg. N <sub>2</sub>	Per gm. fresh tissue	Per mg. N <sub>2</sub>
$\gamma$	$\gamma$	$\gamma$	$\gamma$
124	8.84		
90	6.58	32	2.33
138	10.06	44	3.21
134	9.80	22.5	2.01

TABLE IX  
*Diphosphothiamine Content of Bone Marrow*

Per gm. fresh tissue	Per mg. N <sub>2</sub>
$\gamma$	$\gamma$
3.09	0.246
2.96	0.233
2.30	0.157
2.46	0.177
	0.128
1.72	0.122
1.85	0.129
2.02	0.143
1.70	0.119
Average ..... 2.26	0.161

for 5 minutes at 100° increased the O<sub>2</sub> uptake of bone marrow slices by 42 per cent (Fig. 4). This fluid contained 0.5 mg. of N<sub>2</sub> per cc. and gave positive sulfosalicylic acid and biuret tests. The fluid contained no succinic acid. The ash of whole serum was inactive. We were unable to isolate the active constituent by barium or alcohol precipitation. The active material passed completely through a heavy cellophane membrane. Attempts to isolate it by adsorption on ion exchange resins failed (the resins used were Amberlite IR-4 and IR-100 made by The Resinous Products and

Chemical Company). The active material was extracted by ether. After completion of this work, Warren (18) published the results of his attempts to isolate this active principle.

Boiled yeast juice increased the  $O_2$  uptake of bone marrow by 80 per cent. A mixture of diphosphopyridine nucleotide, diphosphothiamine, and adenosine triphosphate caused only 10 per cent increase in the respiration.

Folic acid, as the sodium salt (150  $\gamma$ ), had no effect on the respiration of bone marrow.

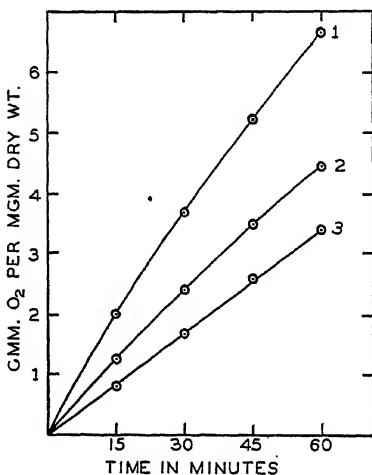


FIG. 4. Effect of serum ultrafiltrate and boiled yeast juice on respiration of rabbit bone marrow slices. Curve 1, boiled yeast juice; Curve 2, serum ultrafiltrate; Curve 3, control.

#### SUMMARY

Although the bone marrow is the site of great cellular activity, the studies reported in this paper have not revealed comparable metabolic activity. The  $QO_2$  values (3.6) resemble those of tissues with low respiration, and, of the numerous substrates added, only glucose, pyruvate, and acetate were utilized vigorously. The large utilization of pyruvate in  $O_2$  as compared with its utilization in  $N_2$  and powerful acetate metabolism are strong indication that carbohydrate utilization in the bone marrow is directed in the presence of  $O_2$  towards the oxidation of pyruvate to acetate and further utilization of this fatty acid. Inhibition of respiration by fluoroacetate is further proof of this contention. There was some synthesis

of citric acid, and aconitase was present in the tissue. The respiration was partially inhibited by malonate, an indication that some portion of the respiration goes through the tricarboxylic acid cycle of Krebs. It is difficult to explain the failure of bone marrow to utilize glutamate adequately. The diphosphopyridine nucleotide content of the bone marrow (121  $\gamma$  per gm.) compared favorably with that found in other tissues, although the diphosphothiamine content (2.26  $\gamma$  per gm.) was rather low. The respiration of bone marrow was increased on addition of boiled yeast juice; folic acid had no effect.

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# INFLUENCE OF INGESTION OF SINGLE AMINO ACIDS ON THE BLOOD LEVEL OF FREE AMINO ACIDS\*

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Our studies of normal and abnormal subjects have demonstrated that the postabsorptive levels of the free amino acids of the plasma remain within rather narrow normal limits except in very extreme terminal conditions. This investigation was made to determine whether the levels of all of the amino acids in the plasma are maintained by the same mechanism and whether there are any cross-relationships between the various amino acids. By feeding a large dose of a single amino acid, the plasma level of that amino acid is raised much above normal. This disturbance of the normal blood pattern gives some indication of the interrelationships existing between the free amino acids of the plasma.

Earlier workers have made similar studies of the effect of single amino acids on the blood amino nitrogen level, but no studies have been made of the effect of ingestion of one amino acid on the level of the other individual amino acids in the blood.

Seth and Luck (1) fed by gavage various amino acids in water to rabbits in amounts equivalent to 560 mg. of amino nitrogen per kilo. They found that glycine, alanine, and histidine caused marked increases in amino nitrogen, whereas leucine, tryptophan, glutamic acid, aspartic acid, and cystine caused very slight if any increases. In a later study with rats Luck (2) found that when glycine or alanine was given to rats there was a marked increase in amino nitrogen, whereas the dicarboxylic acid fraction, the hexone base fraction, and the monoamino acid fraction from casein caused no increase in amino nitrogen. Bang (3) found no increase in the blood amino nitrogen level of rats fed leucine by mouth. Johnston and Lewis (4) working with rabbits found increased blood amino nitrogen levels following glycine, DL-alanine, and glutamic acid feeding but no change following aspartic acid, arginine, and lysine feeding. Shambaugh, Lewis, and Tourtellotte (5) gave 1 gm. of tyrosine and 0.91 gm. of phenylalanine per kilo to rabbits and found no rise in the amino acid nitrogen of the blood at any period, although an increase in phenol content was noted. King and Rappaport (6) injected tyrosine intravenously and found that 95 to 98 per

\* A portion of this study was supported by a grant from the Nutrition Foundation.

cent disappeared within 5 minutes. They indicated that this rapid decrease was due to disappearance into the tissues, since no increase in blood urea or urinary nitrogen sufficient to explain the loss was found. Friedberg and Greenberg (7) recently showed that intravenously administered amino acids are rapidly removed from the blood plasma, but at different rates. They also found that the injected amino acids were concentrated at different rates by the various tissues examined.

## EXPERIMENTAL

### *Method*

Four dogs were used for the series of experiments. Dog 1, a female, weighed 9.0 kilos, Dog 2, male, weighed 12 kilos, Dog 3, male, weighed 12 kilos, and Dog 4, male, weighed 8.7 kilos. The dogs were fasted for 24 hours previous to the experimental day. A 45 cc. sample of blood was removed from the femoral artery with a syringe containing heparin. The amino acid was then given by stomach tube in a neutralized solution or suspension. At intervals of 60, 180, 300, and 1440 minutes, additional blood samples were removed. A 2 week rest interval was allowed before the dog was used again. The doses were given at the levels indicated in Tables I to VI. All experiments were done at least twice, although only typical data from one experiment are reported.

The plasma was analyzed for the various amino acids by the procedures previously described for tryptophan, leucine, isoleucine, valine, arginine, phenylalanine, tyrosine, histidine, and lysine (8). Threonine and methionine were determined with *Streptococcus faecalis* with the media of Stokes *et al.* (9). Cystine was determined with *Leuconostoc mesenteroides* P-60 with Medium D described by Dunn *et al.* (10).

### *Observations*

The plasma levels of leucine, isoleucine, threonine, and valine are found to rise rapidly following ingestion of the dose, reaching a maximum in about 60 minutes, as shown in Tables I and II. After 24 hours the plasma levels are only slightly above normal for these amino acids. When the leucine level is high, there is a fall in the level of all of the other amino acids except cystine, histidine, lysine, and tryptophan. When isoleucine is fed, the plasma level of arginine falls slightly, probably not significantly, while that of phenylalanine, tyrosine, and valine falls significantly. The levels of the other amino acids show no marked change. The high plasma level of threonine and valine reached following ingestion of these amino acids does not appear to affect the level of the other amino acids in the plasma.

TABLE I

*Effect of Ingestion by Dogs of Leucine and Isoleucine on Free Amino Acids of Blood*

The values are reported in micrograms per cc. of plasma. Throughout the tables the bold-faced figures emphasize the blood level of ingested amino acids.

Time after dose	Arginine	Cystine	Histidine	Isoleucine	Leucine	Lysine	Methionine	Phenylalanine	Threonine	Tryptophan	Tyrosine	Valine
Dog 2. L-Leucine, 0.28 gm. per kilo												
min.												
0	35.1	11.7	17.4	19.4	<b>39.2</b>	29.4	14.3	25.0	31.2	14.1	17.7	40.5
60	28.8	9.6	13.8	9.6	<b>334</b>	27.6	7.7	13.0	26.7	12.0	7.5	27.0
180	16.2	9.0	12.3	8.6	<b>168</b>	20.9	5.3	11.9	17.7	11.1	6.0	16.0
300	15.9	10.5	12.6	11.7	<b>86.0</b>	20.6	7.5	15.2	18.8	13.2	7.8	20.1
1440	22.5	12.6	12.6	22.7	<b>61.1</b>	30.6	10.5	14.1	25.0	12.6	10.8	38.9
Dog 4. DL-Isoleucine, 0.56 gm. per kilo												
0	45.9	6.6	12.6	<b>14.3</b>	20.6	29.7	8.6	16.7	18.0	10.5	9.3	24.2
60	38.4	8.1	11.4	<b>305</b>	15.5	23.1	7.0	9.0	15.8	8.7	3.3	17.1
180	36.9	6.9	9.6	<b>183</b>	13.4	25.7	5.9	10.5	15.2	9.6	4.2	14.0
300	33.3	7.8	9.3	<b>115</b>	13.9	28.8	6.5	11.4	14.0	9.3	3.9	14.7
1440	33.0	9.0	9.6	<b>29.3</b>	25.7	31.7	8.9	14.3	18.2	9.9	6.0	32.0

TABLE II

*Effect of Ingestion by Dogs of Threonine and Valine on Free Amino Acids of Blood*

The values are reported in micrograms per cc. of plasma.

Time after dose	Arginine	Cystine	Histidine	Isoleucine	Leucine	Lysine	Methionine	Phenylalanine	Threonine	Tryptophan	Tyrosine	Valine
Dog 3. DL-Threonine, 0.56 gm. per kilo												
min.												
0	24.6	5.4	10.5	19.4	23.3	21.6	10.5	12.6	<b>18.5</b>	8.7	10.5	21.0
60	21.0	5.7	10.2	14.3	19.8	15.0	9.2	9.9	<b>653</b>	8.1	7.5	19.8
180	19.2	6.9	10.2	13.8	21.5	17.6	9.2	10.5	<b>268</b>	7.2	9.0	19.7
300	21.6	6.6	9.9	14.8	21.9	20.4	9.9	11.4	<b>193</b>	7.5	10.2	18.2
1440	22.8	7.8	8.5	16.0	22.5	24.2	8.9	10.2	<b>54.0</b>	5.7	8.7	20.4
Dog 4. DL-Valine, 0.56 gm. per kilo												
0	44.1	7.8	9.9	18.2	32.4	28.6	8.4	12.5	23.0	10.8	9.6	<b>28.8</b>
60	42.9	7.5	9.8	14.7	27.8	25.9	8.2	10.5	19.4	10.2	7.2	<b>623</b>
180	37.5	6.6	9.0	14.6	25.8	20.3	8.0	8.7	23.3	9.3	6.3	<b>548</b>
300	38.7	7.5	8.4	19.5	30.8	21.3	8.4	9.6	20.0	10.5	6.7	<b>488</b>
1440	38.7	9.0	7.1	24.0	33.3	29.3	9.2	9.6	18.0	9.6	7.2	<b>72.2</b>



Table III shows the results of feeding L-arginine, L-histidine, and DL-tryptophan. When arginine is ingested, the tyrosine level may be seen to fall slightly, while no other amino acid is affected, even though the plasma level of arginine increases about 17 times. When the same quantity of histidine is given, the histidine level rises about 100 times. Despite this

TABLE III'

*Effect of Ingestion by Dogs of Arginine, Histidine, and Tryptophan on Free Amino Acids of Blood*

The values are reported in micrograms per cc. of plasma.

Time after dose	Arginine	Cystine	Histidine	Iso-leucine	Leucine	Lysine	Methionine	Phenylalanine	Threonine	Tryptophan	Tyrosine	Valine
Dog 1. L-Arginine, 0.56 gm. per kilo												
<i>min.</i>												
0	17.7		12.3	13.7	16.7	19.7		10.4	32.9	6.3	6.8	23.0
60	297		11.1	10.6	12.2	23.8		8.6	34.8	6.3	4.2	23.4
120	183		12.3	13.9	17.2	18.0		9.2	30.3	7.0	5.1	25.5
300	78.0		10.0	15.8	17.7	18.0		8.7	28.5	5.0	4.2	27.5
1440	25.0		10.0	20.0	21.8	22.3		10.9	38.1	5.7	6.9	34.4
Dog 1. L-Histidine, 0.56 gm. per kilo												
0	24.6		10.8	15.8	16.4	16.4		9.2	27.6	5.7	6.9	21.0
60	28.8		1160	17.6	19.8	26.6		8.9	40.5	7.5	6.3	23.9
120	18.6		338	16.4	16.5	15.5		9.0	31.7	8.0	5.7	33.2
300	21.0		97.5	19.4	20.9	19.5		10.5	33.6	6.3	6.0	29.9
1440	26.4		13.2	24.8	27.5	25.0		12.0	40.7	6.9	6.3	23.0
Dog 3. DL-Tryptophan, 0.56 gm. per kilo												
0	34.5	8.1	9.8	11.8	12.0	22.2	8.0	13.0	11.7	8.7	7.8	15.5
60	28.2	5.1	9.6	11.4	12.2	19.8	8.0	9.8	11.3	110	6.9	16.0
120	28.5	6.0	8.7	15.2	15.2	19.8	7.4	11.5	10.8	154	8.4	18.0
300	27.9	5.4	8.7	13.0	15.3	18.3	7.7	9.5	10.5	95	7.2	17.1
1440	32.7	7.2	8.4	14.6	15.5	28.7	8.3	12.6	12.5	10.5	7.8	18.0

increase in the plasma histidine, there is no effect on the level of any other amino acid. Tryptophan ingestion, similarly, is without effect on any other amino acid level, although the tryptophan content of the plasma is increased about 10 times.

When DL-methionine is given, as shown in Table IV, the level of methionine in the plasma goes up and remains high, even after 24 hours, which is not found to be the case with the other amino acids. As a result of the high plasma level of methionine, there is a lowering of the plasma level of iso-

TABLE IV

*Effect of Ingestion by Dog of Methionine and Cystine on Free Amino Acids of Blood*

The values are reported in micrograms per cc. of plasma.

Time after dose	Arginine	Cystine	Histidine	Iso-leucine	Leucine	Lysine	Methionine	Phenylalanine	Threonine	Tryptophan	Tyrosine	Valine
Dog 1. DL-Methionine, 0.56 gm. per kilo												
min.												
0	42.6	14.7	14.4	17.7	33.5	24.3	16.4	19.2	31.2	24.3	12.0	27.2
60	39.0	20.4	15.3	9.3	20.0	20.6	756	11.3	33.3	19.8	10.7	19.5
180	32.1	15.0	14.1	7.5	14.1	18.9	700	11.3	27.9	16.5	8.7	16.2
300	32.9	13.5	13.8	9.0	20.1	25.0	597	11.7	29.1	15.3	8.4	16.0
1440	37.2	23.1	11.4	14.7	20.0	34.5	429	13.5	28.9	15.3	9.3	22.4
Dog 1. L-Cystine, 0.56 gm. per kilo												
0	38.7	12.7	19.5	21.0	35.4	35.7	15.6	11.4	25.5	14.1	20.1	28.0
60	31.6	12.9	18.7	20.1	34.1	31.2	14.8	11.0	22.1	12.0	19.1	27.5
180	32.3	13.8	16.8	22.2	34.7	29.7	14.8	11.6	20.9	9.0	16.8	29.0
300	26.1	13.8	16.5	19.8	28.5	27.0	13.2	9.6	18.9	7.5	14.7	27.9
1440	27.0	19.5	15.3	26.0	41.6	34.3	15.4	13.7	27.0	12.0	23.4	28.7

TABLE V

*Effect of Ingestion by Dogs of Phenylalanine and Tyrosine on Free Amino Acids of Blood*

The values are reported in terms of micrograms per cc.

Time after dose	Arginine	Cystine	Histidine	Iso-leucine	Leucine	Lysine	Methionine	Phenylalanine	Threonine	Tryptophan	Tyrosine	Valine
Dog 4. DL-Phenylalanine, 0.56 gm. per kilo												
min.												
0	52.2	9.0	13.8	19.0	12.0	22.5	12.9	16.0	22.7	7.8	14.2	24.0
60	48.3	10.5	15.3	14.6	13.0	20.1	12.3	49.2	22.2	6.9	71.4	28.4
180	33.6	10.8	15.0	21.9	16.8	18.9	13.4	28.4	16.8	8.1	24.6	27.0
300	29.1	9.6	14.7	27.0	14.0	18.3	13.7	24.5	16.9	10.8	16.5	21.2
1440	48.0	9.0	12.8	32.0	12.9	24.0	14.1	19.1	27.9	9.0	12.3	23.2
Dog 1. L-Tyrosine, 0.56 gm. per kilo												
0	47.1	4.7	15.0	22.0	34.8	28.8	11.3	15.2	22.0	17.4	16.5	35.9
60	45.9	5.1	14.7	22.1	25.4	28.8	11.7	12.2	21.6	20.4	78.0	30.6
180	44.1	5.4	15.3	24.5	28.3	30.8	12.2	12.6	18.6	19.2	97.5	32.3
300	36.9	5.4	15.0	26.8	28.9	34.9	10.8	12.3	20.7	17.1	60.0	33.8
1440	45.9	6.5	12.3	24.9	30.0	35.5	11.1	14.1	18.3	18.5	12.9	31.2

leucine, phenylalanine, tyrosine, and valine. Arginine and tryptophan show more variable changes. Despite the high level of methionine, there is no increase in the cystine level.

The same dog given the same amount of L-cystine as DL-methionine demonstrates no marked rise in the plasma cystine level. The levels of the other amino acids also do not appear to be affected. This is also shown in Table IV.

Table V demonstrates the results of feeding DL-phenylalanine and L-tyrosine. When phenylalanine is given, the phenylalanine plasma level rises rapidly, while simultaneously there is an increase in the tyrosine level. No other amino acids are affected. When tyrosine is given, the tyrosine level rises without affecting that of phenylalanine or any other amino acid.

#### DISCUSSION

The plasma level of all of the amino acids given, except cystine, was found to rise significantly following ingestion of the corresponding amino acid. Usually the maximum level was reached in about 60 minutes and was followed by a decline. After 24 hours the level was usually very close to normal. Following methionine ingestion, however, even after 24 hours, the level was still many times above normal.

When the leucine, isoleucine, and methionine plasma level rose owing to ingestion of the respective amino acid, the plasma level of certain other amino acids fell. Table VI shows the results of assay of a 24 hour sample of urine excreted by a dog following a dose of L-leucine compared to the assay of a previous sample of urine after a 24 hour fast. There is no increased excretion of any amino acid except leucine; so that increased loss in the urine does not appear to be the explanation for the drop in the plasma amino acid levels of arginine, isoleucine, methionine, phenylalanine, threonine, tyrosine, and valine. It may be that the same amino acid oxidase or deaminase is involved and that when the organism metabolizes the large amount of leucine, for example, the other amino acids are also removed from the plasma. It is also possible that certain equilibria must be maintained which are disturbed by the increased amount of the single amino acid, resulting in a shift of the amino acids from the plasma to the tissues. Studies with tissue slices would probably help to answer this question.

Phenylalanine when ingested causes a rise in the plasma tyrosine level simultaneously with the increased phenylalanine level, indicating the rapidity with which this well recognized conversion occurs. Tyrosine, as might be expected, does not have any effect on the phenylalanine level.

Ingestion of methionine, which is presumably one source of cystine in the body, does not cause an increase in the plasma level of cystine. This is

not surprising, however, when it is noted that ingestion of a large dose of cystine does not cause the plasma level of cystine to rise. Presumably, the organism can rapidly remove the cystine from the plasma either by excretion, conversion, or storage. Methionine, on the other hand, appears to be difficult to metabolize, since the plasma level remains high for a much longer period following methionine ingestion than with any other amino acid studied.

Brown and Lewis (11), using rabbits, found that the inorganic sulfate content of plasma ultrafiltrates increased more rapidly after ingestion of cystine than after methionine. The organic sulfur reached a higher level and remained high longer after methionine than after cystine. Pirie (12) observed that methionine forms sulfate more slowly than cystine when incubated with tissue slices. The rate of absorption from the intestinal tract is

TABLE VI

*Effect of 0.56 Gm. per Kilo of L-Leucine on Urine Excretion of Amino Acids (Dog 3)*

The values are reported in terms of mg. excreted per 24 hours.

	Arginine	Cystine	Histidine	Isoleucine	Leucine	Lysine	Methionine	Phenylalanine	Threonine	Tryptophan	Tyrosine	Valine	Urine volume
Preceding dose	16.5	26.4	2.0	0	1.5	6.0	3.0	2.3	4.0	15.0	1.5	0.13	cc.
Following dose	16.2	21.3	1.6	0	3.0	3.9	2.5	1.4	3.0	11.8	1.1	0.13	175

probably not a factor in explaining the difference in behavior between cystine and methionine since Chase and Lewis (13) found the rates to be approximately the same for both substances.

#### SUMMARY

The plasma level of arginine, histidine, isoleucine, leucine, methionine, phenylalanine, threonine, tryptophan, tyrosine, and valine rose when the corresponding amino acid was given by gavage to dogs. Leucine, isoleucine, and methionine when at a high level in the plasma caused a fall in the level of certain other amino acids. The tyrosine level was found to rise in the plasma when phenylalanine was ingested, while the reverse did not occur. Although the methionine level of the plasma rose to a marked extent after feeding methionine and was maintained for at least 24 hours, the cystine plasma did not increase because of this. In fact, ingestion of an equivalent amount of cystine caused no rise in the cystine plasma level.

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# AMINO ACIDS IN PLASMA

## A CORRECTION

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In a recent publication on glycine and alanine in blood (1) we referred to work by Hier and Bergeim (2) on the concentrations of ten other amino acids from which we calculated that they comprised 76 per cent of the free  $\alpha$ -amino acid N of plasma. Our attention has been called by Hier to a basic error in our calculation in which we computed total nitrogen instead of  $\alpha$ -amino N. Accordingly, we wish to express our regret that this error occurred, and our gratitude for the opportunity to rectify it.

On the basis of this correction the ten amino acids reported by Hier and Bergeim (2) comprise 47 per cent of the total  $\alpha$ -amino acid N of human plasma. If to this is added 24 per cent in the form of glycine plus alanine (1) and 18 to 25 per cent in the form of glutamine (3), we can account for 89 to 96 per cent of the free  $\alpha$ -amino N. Since, as pointed out by Hier,<sup>1</sup> cystine plus methionine comprises an additional 5 per cent approximately, only a small amount remains to be accounted for in the form of cysteine, hydroxyproline, proline, and aspartic acid. The concentrations of the latter two in plasma are said to be negligible.<sup>1</sup>

It is, therefore, apparent that our inference that the values for the free amino acids in plasma determined microbiologically by Hier and Bergeim (2) are erroneously high is incorrect in view of the revised calculations.

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<sup>1</sup> Hier, S. W., personal communication.



# THE UTILIZATION FOR ANIMAL GROWTH OF TOBACCO MOSAIC VIRUS AS A SOLE SOURCE OF PROTEIN IN THE DIET

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Tobacco mosaic virus can be considered as a pathological nucleoprotein which reaches very high concentrations within the cells of certain plants following infection with the virus. In the case of Turkish tobacco plants, concentrations of tobacco mosaic virus of over 3 mg. per ml. have been found in the juices pressed from macerated plants (1). The large amounts of virus present in infected cells and the fact that the virus persists within the cells throughout the life of the plant indicate that the enzymes of the plant may be unable to bring about the hydrolysis of the virus. The effect of a number of enzymes on purified preparations of tobacco mosaic virus has been studied. Commercial trypsin (2-4), crystalline trypsin (3), pancreatin (4), papain (4), crystalline ribonuclease (5), an autolyzed kidney preparation (4), and intestinal nucleophosphatase (6) were found to show no enzymatic activity towards tobacco mosaic virus. Trypsin and ribonuclease were found to inactivate tobacco mosaic virus, but following the removal of these enzymes the virus activity was recovered (3, 5, 7, 8). Pfankuch and Kausche (9) have reported that phosphatase preparations from turnip and potato inactivated tobacco mosaic virus. Schramm (10) has reported that an intestinal phosphatase splits nucleic acid from tobacco mosaic virus. However, Cohen and Stanley (6) were unable to confirm the latter work. It might also be pointed out that the juices of Turkish tobacco plants are rich in phosphatase and this has not been found to cause the release of phosphate from the virus (11). It is possible that Schramm's results were due not to the action of nucleophosphatase but to hydrolysis caused by the strong trichloroacetic acid used to precipitate the protein. The only enzyme preparation which has been found to cause the irreversible inactivation of tobacco mosaic virus is pepsin (12). It was found in studies with crystalline pepsin that the virus was inactivated only under conditions favorable for proteolytic



activity and that the rate of inactivation varied directly with the concentration of pepsin. However, the rate of peptic digestion of tobacco mosaic virus was found to be much less than the rates of digestion of ordinary proteins.

In view of the apparent resistance of tobacco mosaic virus to many enzymes and because of the fact that the virus is a representative of a most unusual type of material, it appeared of interest to determine whether tobacco mosaic virus could be utilized as a source of protein by animals. The chemical make-up, including the amino acid content of the purified virus, has been studied (13-21). Approximately all of the virus has been accounted for, as can be seen from Table I. The amino acid content of the virus is, in general, similar to that of casein. However, histidine and methionine do not appear to be present in tobacco mosaic virus and the content of lysine appears to be lower than in the case of casein. If the virus could be utilized as food by animals, it seemed possible that confirmation of the presence of certain essential amino acids as well as of the absence of histidine and methionine might be obtained in animal feeding studies. Experiments in which purified tobacco mosaic virus was used as a source of protein for rats are described in the present paper.

The results of these experiments prove that tobacco mosaic virus when supplemented with only three amino acids supports growth of the white rat, thus demonstrating that the other essential amino acids of the virus molecule are available for the metabolism and growth of animals. The digestion of the virus protein must be nearly, if not entirely, complete, since moderate growth is supported by a diet containing only 10 per cent of virus with the addition of amino acids to make up an additional 2.5 per cent.

Only three amino acids, histidine, methionine, and lysine, were required to supplement those supplied by the virus. When either histidine or methionine was omitted from this supplement, a sharp loss in weight of the animal occurred, but when lysine was the only amino acid omitted from the supplement, growth ceased but marked weight loss did not occur. These results are in agreement with the analytical findings that histidine and methionine are entirely absent from the tobacco mosaic virus molecule, while lysine occurs in small amounts (1.47 per cent). Only a partial lysine deficiency, therefore, results when the virus is the only source of this amino acid. At the 20 per cent level of the virus, lysine is present as 0.29 per cent of the diet. Rose (22) has found that the minimum requirement for lysine for growth of the rat is 1.0 per cent of the diet.

It might be pointed out that the presence of a large amount of nucleic acid in the tobacco mosaic virus (5.8 per cent) did not appear to have a deleterious effect.

## EXPERIMENTAL

*Preparation of Purified Tobacco Mosaic Virus*—Several lots of purified tobacco mosaic virus, prepared by chemical as well as by centrifugation methods described earlier (14, 15), were made during the course of several months and combined. The white fluffy material, containing about 8 per

TABLE I  
*Composition of Casein and of Tobacco Mosaic Virus*

Chemical constituent	Casein	Tobacco mosaic virus	
	Per cent by weight*	Per cent by weight†	Bibliographic reference No.
Alanine.....	5.5	2.4 (i), 5.1 (b)	(17, 21)
Amide nitrogen as NH <sub>3</sub> ...		1.9	(17)
Arginine.....	4.3	8.5-9.2 (i) (c), 9.8 (b)	(15, 17-19, 21)
Aspartic acid.....	6.1	2.4-2.8 (i), 13.5 (b)	(17, 21)
Cysteine and cystine.....	0.35	0.60-0.76 (b) (c)	(14, 21)
Glutamic acid.....	23.3	5.1-5.5 (i), 11.3 (b)	(17, 21)
Glycine.....	0.5	0.0 (i) (c), 1.9 (b)	(15, 17, 21)
Histidine.....	2.1	0.0 (b) (c)	(15, 17-19, 21)
Hydroxyproline.....	?	3.3 ?	(15)
Isoleucine.....	6.3	6.6 (b)	(21)
Leucine.....	9.7	6.1 (i), 9.3 (b)	(17, 19, 21)
Lysine.....	7.6	1.47 (b)	(18, 19, 21)
Methionine.....	3.4	0.0 (i) (b)	(14, 19, 21)
Nucleic acid.....		5.8 (i)	(13)
Phenylalanine.....	5.0	5.6-6.7 (c), 8.4 (b)	(15, 16, 19, 21)
Proline.....	7-8	4.2-4.7 (i) (c), 5.8 (b)	(15, 17, 21)
Serine.....	7.7	6.4 (c), 7.2 (b)	(15, 21)
Threonine.....	3.8	5.3 (c), 9.9 (b)	(15, 19, 21)
Tryptophan.....	1.2	1.9-4.6 (c), 2.1 (b)	(15, 16, 19, 21)
Tyrosine.....	6.7	3.8 (c), 3.8 (b)	(15, 16, 20, 21)
Valine.....	6.5	3.9 (i), 9.2 (b)	(17, 19, 21)

\* Hawk, P. B., Oser, B. L., and Summerson, W. H., *Practical physiological chemistry*, Philadelphia, 12th edition, 109 (1947).

† i, b, or c indicates determination was by isolation, microbiological, or colorimetric method, respectively.

cent moisture and obtained following dialysis against distilled water and drying from the frozen state, was the same as that used in various studies on the amino acid content of the virus. Following drying from the frozen state, about two-thirds, by weight, of the virus preparation was found to be insoluble in 0.1 M phosphate buffer at pH 7. The remainder was soluble in this solvent and on examination by means of an electron microscope was found to consist of rods 15 m $\mu$  in width and 280 m $\mu$  or less in length. A

comparison of the amino acid content of tobacco mosaic virus with that of casein is given in Table I. In those instances in which the amount of a given amino acid was determined by colorimetric or microbiological methods in addition to an isolation method, the former values are used, since isolation methods usually yield low results.

*Feeding Experiments*—Albino rats weighing about 100 gm. were fed *ad libitum* on purified diets in which the protein was supplied in the form of pure tobacco mosaic virus supplemented with various combinations of three essential amino acids, methionine, histidine, and lysine. Cystine was also included in the diets so that the low cystine-cysteine content of the protein would not interfere with the interpretation of the results on methionine supplementation. The composition of these diets is given in Table II.

TABLE II  
*Composition of Diets*

	Diet I	Diet II	Diet III	Diet IV	Diet V
	gm.	gm.	gm.	gm.	gm.
Casein.....	22.0				
Tobacco mosaic virus.....		20.0	10.0	20.0	20.0
L-Lysine hydrochloride.....			1.0	1.0	1.7
L-Histidine hydrochloride.....			0.7	0.7	0.7
DL-Methionine.....			0.3	0.3	0.7
L-Cystine.....			0.5	0.5	0.5
NaHCO <sub>3</sub> .....			0.7	0.7	1.1
Crisco.....	19.0	19.0	19.0	19.0	19.0
Corn oil.....	1.0	1.0	1.0	1.0	1.0
Salt mixture (Osborne and Mendel)*.....	4.0	4.0	4.0	4.0	4.0
Sucrose.....	54.0	56.0	62.8	52.8	51.3

\* Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 572 (1919).

The oil-soluble vitamins were included in the corn oil to provide 4000 U. S. P. units of vitamin A, 400 U. S. P. units of vitamin D, 1.0 mg. of *dl*- $\alpha$ -tocopherol, and 100  $\gamma$  of menadione per 100 gm. of diet. The water-soluble vitamins were administered as 1.0 ml. daily of an aqueous solution containing 40  $\gamma$  of thiamine hydrochloride, 40  $\gamma$  of riboflavin, 40  $\gamma$  of nicotinic acid, 40  $\gamma$  of pyridoxine hydrochloride, 200  $\gamma$  of calcium *dl*-pantothenate, 3 mg. of inositol, 1 mg. of *p*-aminobenzoic acid, 25 mg. of choline chloride, 50 mg. of ryzamin-B, and 50 mg. of sucrose.

The general plan of the experiment was as follows: The rat was given the virus-containing diet supplemented with all four amino acids until a satisfactory rate of growth was established. The diet was then changed by the omission of one of the amino acids and substitution of an equal

amount of sucrose. After sufficient time (7 to 14 days) to establish the effect of this change on the rate of growth, the original diet was restored.

The rat resumed growth and after a few days one of the other amino acids was omitted from the supplement.

TABLE III  
*Effect of Virus-Containing Diet on Growth*

Rat No.	Days on diet	Diet No.	Average daily food intake	Total weight change	Average daily weight change
			gm.	gm.	gm.
2661	19	I	13.3	+112	+5.9
2662	19	"	10.5	+ 85	+4.5
2664	47	II	3.8	- 45	-1.0
1550	40	III	8.8	+ 72	+1.8
	13	" Without lysine	7.8	- 10	-0.8
	10	"	9.3	+ 52	+5.2
	7	" Without histidine	4.3	- 14	-2.0
	10	"	16.6	+ 40	+4.0
	10	" Without methionine	5.0	- 19	-1.9
	6	"	9.0	+ 4	+0.7
2659	27	IV	8.1	+ 67	+2.5
	10	" Without lysine	10.0	- 6	-0.6
	5	"	14.0	+ 30	+6.0
	10	" Without cystine	11.1	+ 30	+3.0
2660	21	"	10.5	+ 72	+3.4
	8	" Without methionine	4.6	- 15	-1.9
	7	"	16.0	+ 50	+7.1
	8	" Without histidine	5.6	- 22	-2.7
	8	"	10.6	+ 40	+5.0
2663	7	V	10.7	+ 30	+4.3
	9	" Without histidine	6.9	- 17	-1.9
	5	"	13.0	+ 26	+5.2
	14	" Without lysine	9.3	+ 3	+0.2
	3	"	11.7	+ 24	+8.0
	7	" Without methionine	4.1	- 19	-2.7
	2	"	11.5	+ 12	+6.0

The results of these experiments are given in Table III. Rat 1550 received a diet containing only 10 per cent of virus in order to exaggerate the border line amino acid deficiency in this protein. This animal was given only 10 mg. of choline chloride per day. The other rats received the virus at a higher level, 20 per cent, to support more nearly optimum growth. In Diet V given to Rat 2663, the amounts of lysine and methionine were increased to a level similar to that in a diet containing 22 per cent casein.

Rat 2664 received Diet II containing 20 per cent of virus, but without any amino acid supplementation. Although considerable weight loss occurred, the rat survived for the experimental period of 47 days, at the end of which time it was sacrificed. Two control animals, Rats 2661 and 2662, grew at a satisfactory rate on Diet I, in which casein was substituted for the virus as the source of protein.

#### SUMMARY

The amino acids present in tobacco mosaic virus are utilized by the white rat for growth.

This virus at a level of 20 per cent of the diet supplies all of the amino acid requirements for growth except for histidine, methionine, and lysine.

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## LETTERS TO THE EDITORS

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### AN ACETYLCHOLINE COMPLEX IN NERVOUS TISSUE

Sirs:

It has been observed by a number of investigators that the acetylcholine (Ach) activity of alcohol extracts of nerve tissue increases upon boiling. The significance of this finding has recently been studied in detail by N. O. Abdon and his associates.<sup>1</sup> Their experiments, concerned primarily with skeletal muscle, led them to conclude that Ach is normally present in resting muscle in the form of an inactive complex, which releases free Ach when the muscle functions. During the recovery phase the free Ach is largely removed by direct resynthesis into the complex rather than by esterase hydrolysis. In studying the properties of the complex, Abdon found that it could be precipitated as the barium salt, that it was unstable to boiling or strong acids, maximally stable at about pH 4, soluble in water or alcohol, and insoluble in ether or acetone. He was able to separate the complex from active Ach in alcohol extracts by precipitating it in acetone.

Since this work was done on skeletal and cardiac muscle, with only one experiment on nervous tissue, we attempted to extend the observations to the brain and sciatic nerves of rats. We were unable to achieve a complete separation of the complex from active Ach, although we succeeded in obtaining an acetone precipitate which had a high Ach activity after boiling. The complex was present in two experiments on brain tissue, and in three experiments on peripheral nerves. Eight other experiments were inconclusive. The total amounts of Ach obtained by the cold extraction procedure of Abdon were generally comparable to the amounts obtained by the usual methods of eserinated Ringer extraction of unfrozen tissue.

In the course of this work we found that it is necessary to prevent any thawing of a frozen tissue until complete inactivation of the esterase has been achieved. When the tissue can be homogenized in  $-70^{\circ}$  alcohol in a Waring blender, this presents no serious problem, but unusual precautions must be taken to minimize Ach loss when frozen tissue is to be extracted with an aqueous solution. Frozen rat brain plunged into Ringer's solution at about  $90^{\circ}$  yielded no detectable Ach (determined by bioassay on the ventricle of *Venus mercenaria*); plunged into briskly boiling Ringer's solu-

<sup>1</sup> Abdon, N. O., *Acta pharmacol.*, **1**, 325 (1945). Abdon, N. O., and Hammar skjöld, S. O., *Acta physiol. Scand.*, **8**, 75 (1944).

tion, it yielded 0.8  $\gamma$  of Ach per gm. of tissue and ground to a powder in a mortar packed in solid CO<sub>2</sub> and then plunged into boiling Ringer's solution, it yielded 2.1  $\gamma$  of Ach per gm. Frozen brain ground in eserinated acid Ringer's solution at 25° yielded only 0.4  $\gamma$  of Ach per gm. For comparison, the Ach content of fresh rat brains, as reported in the literature, ranges from about 0.7 to 3  $\gamma$  of Ach per gm. Frozen beef brains, extracted with eserinated Ringer's solution at room temperature, gave only 0.05 and 0.01  $\gamma$  of Ach per gm. in two experiments. The values usually given for the Ach content of beef brain range from 0.2 to 0.9  $\gamma$  of Ach per gm. Since we have shown that considerable Ach synthesis during the extraction procedure may complicate Ach determination on fresh nervous tissue,<sup>2</sup> it would be of value to establish a satisfactory technique for extracting Ach in an aqueous solution from frozen tissues.

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<sup>2</sup> Prajmovsky, M., and Welsh, J. H., *J. Neurophysiol.*, in press.

## THE ANEMIA-PRODUCING PROPERTIES OF $\alpha$ -AMINO- $\epsilon$ -HYDROXYCAPROIC ACID

Sirs:

In the course of a new synthesis of lysine,  $\alpha$ -amino- $\epsilon$ -hydroxycaproic acid was isolated as an intermediary compound.<sup>1</sup> It was shown that it could not replace lysine in the diet of rats and that it was probably toxic. Upon publication of these original findings,<sup>2</sup> Dr. Richard J. Block drew our attention to the probable identity of this compound with the anemia-producing factor known to exist in deaminized casein.<sup>3</sup> This was investigated.

In a first experiment, twenty-one male rats averaging 90 gm. in body weight were put on the following diet: zein 17.4, DL-tryptophan 0.4, soy bean oil 4.0, salts 4.0, cerelose 72, and Cellu flour 2.0 gm. per cent. 100 gm. of ration contained thiamine HCl 0.4, riboflavin 0.5, pyridoxine HCl 0.5, calcium pantothenate 3.0, nicotinic acid 3.0, 2-methyl-1,4-naphthoquinone 0.1, inositol 10, and choline chloride 150 mg. After 10 days, the rats were divided into three groups. Group I received 1.5 per cent  $\alpha$ -amino- $\epsilon$ -hydroxycaproic acid and Group II received 1.5 per cent DL-lysine, these changes being made at the expense of the cerelose; Group III remained on the basal ration. 2 weeks later average hemoglobin values were 8.8, 14.2, and 13.5 gm. per 100 ml. of blood, respectively. Red blood cell counts were as follows: Group I, substituted lysine 2,880,000; Group II, DL-lysine 7,790,000, and Group III, no lysine 7,700,000. Body weight changes during these 2 weeks were -14, +36, and -8 gm., respectively.

In a second experiment, 18 per cent casein was substituted for the zein-tryptophan fraction of the previous ration and the aminohydroxycaproic acid content was lowered to 1 per cent. Sixteen rats were put on this diet and ten on a control ration. After 17 days, the experimental rats showed an average hemoglobin value of 10.8 gm. per cent and a red blood cell count of 5,610,000 per c.mm., as compared with 13.5 and 8,360,000, respectively, for the control rats.

The experimental rats were then divided into four groups, one of them remaining on the previous ration and the other three receiving 1 per cent of a liver concentrate 1:20, 1 per cent L-lysine, and daily injections of 100  $\gamma$  of folic acid, respectively. None of these corrective measures proved successful. Average values, 2 weeks later, were hemoglobin 5.5 gm., red blood cells 4,550,000 per c.mm., and red blood cell volume 26 per cent. The

<sup>1</sup> Gaudry, R., to be published.

<sup>2</sup> Gingras, R., Pagé, E., and Gaudry, R., *Science*, **105**, 621 (1947).

<sup>3</sup> Hogan, A. G., Powell, E. L., and Guerrant, R. E., *J. Biol. Chem.*, **137**, 41 (1941).



possibility remains, however, that a favorable response might have been obtained with a lesser amount of the toxic compound in the ration.

While these experiments were in progress, Dr. Charles E. Dent, who had asked for a sample of  $\alpha$ -amino- $\epsilon$ -hydroxycaproic acid, kindly informed us that its paper chromatogram was the same as that of a substance he had isolated from deaminized casein. It thus appears that the compound we have studied is responsible for the anemia produced in rats fed deaminized casein. On the other hand, we have failed to observe any anemia in rats maintained for 24 days on a lysine-free ration. This is in contrast to the findings of Hogan *et al.*<sup>3</sup> and of Gillespie, Neuberger, and Webster,<sup>4</sup> but is probably due to the short duration of our experiment.

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<sup>4</sup> Gillespie, M., Neuberger, A., and Webster, T. A., *Biochem. J.*, **39**, 203 (1945).

# THE INVOLVEMENT OF COENZYME A IN ACETATE OXIDATION IN YEAST\*

Sirs:

Recently, a participation was reported of the pantothenic acid derivative, coenzyme A (Co A), in enzymatic acetylation<sup>1</sup> and in pyruvic acid oxidation.<sup>2</sup> At this stage the exploration of acetate breakdown in yeast seemed promising, as the early work by Roger Williams and his collaborators<sup>3</sup> indicated already in general a function of pantothenic acid in carbohydrate metabolism. *Saccharomyces cerevisiae*, strain LK2G12, obtained from Dr. John Reiner, Washington University, was used in the present work. The following procedure was adopted. Samples of pantothenic acid-deficient yeast were divided into two parts. Both were aerated in a glucose-phosphate medium for 1½ hours, with and without pantothenic acid, and washed twice with water. The pantothenic acid-treated yeast contained now around 400 units per gm. of dry weight of Co A against 100 to 150 units in the deficient sample.

Substrate	Time	Pretreated with pantothenate and glucose, Co A .370 γ per gm.		Pretreated with glucose alone, Co A 135 γ per gm.	
		O <sub>2</sub> consumed	Acetic acid remaining	O <sub>2</sub> consumed	Acetic acid remaining
	<i>min.</i>	<i>microliters</i>	<i>micromoles</i>	<i>microliters</i>	<i>micromoles</i>
Acetic acid, 20 micromoles added at time 0	40	137	15.7	109	16.6
	100	382	9.8	284	13.3
	165	740	0.1	417	10.5
Ethanol, 20 micromoles added at time 0	40	172	4.1	103	4.8
	100	531	4.4	310	8.0
	160	814	0.0	443	11.5

Each vessel contained 43 mg. of dry weight cells in 3 ml. of 0.02 M KH<sub>2</sub>PO<sub>4</sub>; air as gas phase; temperature 37°.

It appears that the high Co A yeast respired acetate and ethanol about twice as fast as the deficient sample. Acetate determination<sup>4</sup> showed furthermore that, at a time when all the acetate was used in the high Co A

\* This work was supported by a grant from the Commonwealth Fund.

<sup>1</sup> Lipmann, F., Kaplan, N. O., Novelli, G. D., Tuttle, L. C., and Guirard, B. M., *J. Biol. Chem.*, **167**, 869 (1947).

<sup>2</sup> Novelli, G. D., and Lipmann, F., *Arch. Biochem.*, **14**, 23 (1947).

<sup>3</sup> Williams, R. J., Mosher, W. A., and Rohrman, E., *Biochem. J.*, **30**, 2036 (1936). Teague, P. C., and Williams, R. J., *J. Gen. Physiol.*, **25**, 777 (1942).

<sup>4</sup> Acetate was determined by an unpublished enzymatic micromethod, applying the liver system for sulfanilamide acetylation (Soodak, M., and Lipmann, F.).

yeast, only about half of it had disappeared in the deficient yeast. With ethanol as a substrate, more than half its equivalent accumulated as acetic acid in the deficient yeast, while only transient accumulation was observed in the Co A-rich sample. Less accumulation of acetate occurred with glucose in deficient yeast. The experiments support the view that Co A is concerned with the primary attack on acetate, presumably the condensation with oxalacetate.

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# THE IN VIVO OXIDATION OF URIC ACID\*

Sirs:

We have fed uric acid, labeled with isotopic nitrogen in the 1 and 3 positions, to Sherman strain rats at a level of 249 mg. per kilo of body weight per day for 3 days and have found that, as in the case of adenine labeled in the 1 and 3 positions,<sup>1</sup> there results a uniform distribution of the isotopic nitrogen between the hydantoin and the urea moieties of the urinary allantoin formed. This demonstrates for the *in vivo* oxidation of uric acid a mechanism involving a symmetrical intermediate,<sup>2</sup> and a parallelism to the *in vitro* oxidation with alkaline permanganate. The negligible concentra-

	Atom per cent, N <sup>15</sup> excess	Calculated on basis of 100 per cent in uric acid fed
Uric acid (dietary).....	16.0	100
Copper purines (viscera).....	0.000	0.00
Urea.....	0.006	0.04
Allantoin.....	5.37	33.4
Hydantoin (derived from allantoin).....	5.34	

tion of isotopic nitrogen found in the urinary urea shows that degradation of this uric acid to either ammonia or urea does not occur in the rat. The copper purines isolated from the viscera contained no isotopic nitrogen.

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